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(54) Title: MULTIMERIC FORMS OF HUMAN RHINOVIRUS RECEPTOR PROTEIN

(57) Abstract

The present invention relates to novel forms and configurations of intercellular adhesion molecule (ICAM) including multimeric configurations that effectively bind to human rhinovirus and can effectively reduce HRV infectivity. When in a multimeric configuration, preferably as dimers, these proteins display enhanced binding of HRV and are able to reduce HRV infectivity as well as the infectivity of other viruses known to bind to the "major" group human rhinovirus receptor (HRR). The multimerized proteins may also be used to block tICAM interaction with lymphocyte function-associated antigen-1 (LFA-1).

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MULTIMERIC FORMS OF HUMAN RHINOVIRUS RECEPTOR PROTEIN

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of copending application USSN 07/704,984 (filed 24 May 1991), which in turn is a continuation-in-part of copending application USSN 07/556,238 (filed 20 July 1990).

The present invention relates to novel forms and multimeric configurations of intercellular adhesion molecule (ICAM), including both full-length and truncated forms of these proteins, that effectively bind to human rhinovirus and can effectively reduce HRV infectivity, and to methods of making and using same.

Full-length ICAM, also known as human rhinovirus receptor (HRR), is termed transmembrane ICAM (tmICAM-1); non-transmembrane ICAM forms, also known as truncated ICAM (tICAM), are less than full length. When in a multimeric configuration, preferably as dimers, these proteins display enhanced binding of human rhinovirus (HRV) and are able to reduce HRV infectivity. In addition, these multimerized proteins may also be used to reduce infectivity of other viruses that are known to bind to the 'major' group human rhinovirus receptor (HRR), such as Coxsackie A virus, and may also be used to block transmembrane intercellular adhesion molecule (tmICAM) interaction with lymphocyte function-associated antigen-1 (LFA-1), which is critical to many cell adhesion processes involved in the immunological response. Lastly, these multimerized proteins may be used to study the ICAM-1/HRV interaction especially with respect to designing other drugs directed at affecting this interaction.

Human rhinoviruses are the major causative agent of the common cold. They belong to the picornavirus family and can be classified based on the host cell receptor to which they bind. Tomassini, et al., J. Virol., 58: 290 (1986) reported the isolation of a receptor protein involved in the cell attachment of human rhinovirus. Approximately 90% of the more than 115 serotypes of rhinoviruses, as well as several types of Coxsackie A virus, bind to a single common receptor termed the "major" human rhinovirus receptor (HRR); the remaining 10% bind to one or more other cell receptors.

Recently, Greve, J. et al., Cell, 56:839 (1989), co-authored by the co-inventors herein, identified the major HRR as a glycoprotein with an apparent molecular mass of 95,000 daltons and having an amino acid sequence essentially identical to that deduced from the nucleotide sequence of a previously described cell surface protein named intercellular adhesion molecule (ICAM-1) [see Fig. 1; Simmons, D. et al., Nature, 331:624 (1988); Staunton, et al., Cell, 52:925-933 (1988)]. Subsequently, Staunton, D.E., et al., Cell, 56:849 (1989), confirmed that ICAM-1 is the major surface receptor for HRV. See also, Staunton, et al., Cell, 61:243-254 (1990). ICAM-1 is an integral membrane protein 505 amino acids long and has: i) five immunoglobulin-like extracellular domains at the amino-terminal end (amino acid residues 1-453), ii) a hydrophobic transmembrane domain (454-477), and iii) a short cytoplasmic domain at the carboxy-terminal end (478-505). See Fig. 2. ICAM-1 is a member of the immunoglobulin supergene family and functions as a ligand for the leukocyte molecule, lymphocyte function associated molecule-1 (LFA-1), a member of the integrin family. Heterotypic binding of LFA-1 to ICAM-1 mediates cellular adhesion of diverse cell types and is important in a broad range of immune interactions; induction of ICAM-1 expression by cytokines during the inflammatory response may regulate leukocyte localization to inflammatory sites. The primary structure of ICAM-1 has been found to be homologous to two cellular adhesion molecules, i.e., neural cell adhesion molecule (NCAM) and myelin-associated glycoprotein (MAG).

Several approaches to decreasing infectivity of viruses in general, and of rhinovirus in particular, have been pursued including: i) developing antibody to the cell surface receptor for use in blocking viral binding to the cell, ii) using interferon to promote an anti-viral state in host cells; iii) developing various agents to inhibit viral replication; iv) developing antibodies to viral capsid proteins/peptides; and v) blocking viral infection with isolated cell surface receptor protein that specifically blocks the viral binding domain of the cell surface receptor.

Using this last approach, Greve, et al., Cell, 56:879 (1989), supra, reported that purified tmICAM-1 could bind to rhinovirus HRV3 in vitro. Unpublished results with HRV2, HRV3, and HRV14 demonstrate a positive correlation between the ability

to bind to rhinovirus and the ability to neutralize rhinovirus particularly if the binding studies are carried out under conditions where ICAM-1 is presented in a particular form and configuration as discussed further, infra. Results (unpublished) using HRV14 and HRV2 demonstrate a positive correlation between the receptor class of the virus and the ability to bind to tmICAM-1 in vitro. That is, ICAM-1, being the major receptor, can bind to HRV3, HRV14, and other "major" receptor serotypes and neutralize them, while it does not bind or neutralize HRV2, a "minor" receptor serotype. Further studies (unpublished), using purified tmICAM-1, demonstrate that it effectively inhibits rhinovirus infectivity in a plaque-reduction assay when the rhinovirus is pretreated with tmICAM-1 (50% reduction of titer at 10 nM receptor and one log reduction of titer at 100 nM receptor protein). These data were consistent with the affinity of rhinovirus for ICAM-1 of Hela cells, which had an apparent dissociation constant of 10 nM, and indicated a direct relationship between the ability of the receptor to bind to the virus and to neutralize the virus. Because large-scale production of tmICAM-1 is not presently economically feasible, and because maintenance of tmICAM-1 in an active form requires the use of detergents, alternate means of producing a receptor protein for use as a rhinovirus inhibitor are desirable. Forms of the tmICAM-1 cDNA gene have been developed (as well as cell lines that produce the expression products; USSN 07/390,662) that have been genetically altered to produce truncated ICAM-1 molecules. See Fig. 2. These truncated forms of ICAM-1 (tICAM(453) and tICAM(185)) lack the transmembrane region and are secreted into the cell culture medium. They bind to rhinovirus in the assay described in Greve, et al., Cell, 56:879 (1989), supra, although at substantially reduced levels relative to tmICAM-1. Thus, their effectiveness as inhibitors of rhinoviral infectivity appeared to be less than that of tmICAM-1. See generally co-pending applications USSN 07/239,571; USSN 07/262,428; USSN 07/678,909; USSN 07/631,313; USSN 07/301,192; USSN 07/449,356; USSN 07/798,267; USSN 07/556,238; USSN 07/704,996; and USSN 07/704,984.

USSN 07/239,571 filed September 1, 1988, and its CIP applications USSN 07/262,428, USSN 07/390,662 (abandoned in favor of continuation USSN 07/678,909), USSN 07/631,313, and USSN 07/704,996 are directed to the use of

transmembrane rhinovirus receptor as an inhibitor of rhinovirus infectivity using non-ionic detergent to maintain the transmembrane protein in solution, and directed to truncated intercellular adhesion molecules (tICAM) comprising one or more of the extracellular domains I, II, III, IV, and V of tmICAM, which truncated forms do not require the presence of non-ionic detergent for solubilization (see Fig. 2).

USSN 07/130,378 filed December 8, 1987 (abandoned in favor of continuation application USSN 07/798,267), and CIP application USSN 07/262,570 (now abandoned) are directed to transfected non-human mammalian cell lines which express the major rhinovirus receptor (HRR) and to the identification of HRR as intercellular adhesion molecule.

USSN 07/301,192, filed January 24, 1989, and its CIP application USSN 07/449,356 are directed to a naturally- occurring soluble ICAM (sICAM) related to but distinct from tmICAM in that this sICAM lacks the amino acids spanning the transmembrane region and the cytoplasmic region; in addition this sICAM has a novel sequence of 11 amino acids at the C-terminus.

Subsequently, Marlin, S.D., et al., Nature, 344:70 (1990), reported the construction and purification of a truncated soluble form of the normally membrane-bound ICAM-1 molecule which they termed sICAM-1. It has both the transmembrane domain and the cytoplasmic domain of the protein deleted and differs from the wild-type amino acid sequence by a single conservative substitution at its carboxyl end. It is composed of residues 1-452 of ICAM-1 plus a novel phenylalanine residue at the C-terminus. These workers demonstrated that sICAM-1 was required at levels >50 μg/ml to prevent the binding of HRV14 virus to cells. However, they also found that sICAM-1 at 1 μg/ml (18 nM), when continually present in the culture medium, was able to inhibit by 50% the progression of an infection by HRV54. The inhibitory activity was correlated with the receptor class of the virus, in that Coxsackie A13 but not poliovirus or HRV2 was inhibited; infectivity data for HRV14 was not reported, however. Thus, they did not demonstrate a direct correlation between binding and inhibition of infectivity. Further, as discussed in greater detail, infra, attempts to reproduce the results obtained by Marlin, et al. have not been successful.

To date, no one has been able to demonstrate an agent that binds to and effectively reduces infectivity of human rhinovirus (by blocking viral infection with isolated cell surface receptor protein) as effectively as tmICAM-1; accordingly there continues to exist a need in the art for a form of ICAM-1 that can effectively bind to human rhinovirus and can effectively reduce HRV infectivity.

BRIEF SUMMARY OF THE INVENTION

Provided by the invention are multimeric configurations of transmembrane ICAM (tmICAM-1) and multimeric configurations of non-transmembrane ICAMs (tICAMs), having improved rhinovirus binding and inhibition activity.

As noted, supra, tmICAM-1 isolated from mammalian cells has the capacity to neutralize human rhinoviruses belonging to the major receptor group, but only if maintained in solution with detergent. Certain soluble fragments of ICAM-1 have been found to have a reduced capacity for binding virus and do not reduce infectivity as effectively as tmICAM-1. To date, no one has been able to ascertain the reason for this reduced capacity.

It has been proposed by others that the rhinovirus receptor exists on cells in a pentameric form [Tomassini, J., and Colonno, R., J. Virol., 58:290-295 (1986)]. However, quantitation (unpublished results of the co-inventors herein) of the rhinovirus and anti-ICAM-1 monoclonal antibody (Mab) binding to HeLa cells has revealed a maximum of 30,000 virions bound per cell (determined by the binding of [^{35}S]methionine-labeled HRV) and 50,000-60,000 ICAM-1 molecules per cell (determined by the binding of radio-labeled Mab to ICAM-1). These results prompted further studies to examine the possibility that rather than five, only between one and two ICAM-1 molecules on the surface of cells are bound per HRV particle bound to the cell.

Genetically engineered forms of truncated ICAM-1 that lack the C-terminal transmembrane domain are secreted into the culture medium of mammalian cells transfected with the recombinant gene. The purification of such secreted ICAM molecules from spent culture medium of cells stably transfected with the genes

therefor is described herein. In a solution-HRV binding assay and in an HRV neutralization assay, it was found that the monomeric forms tend to have substantially reduced avidity for HRV relative to tmICAM-1. However, it has now been discovered that when such tICAMs are presented in multimeric form and then incubated with HRV, the virus-binding activity of the multimeric tICAMs becomes comparable to that of tmICAM-1. This binding of multimeric tICAMs to HRV has the same properties as the binding of HRV to ICAM-1 on HeLa cells: it is inhibited by anti-ICAM-1 Mabs, it is specific for rhinoviruses of the major receptor group, and has the same temperature dependence as the binding of rhinovirus to cells (i.e., binds well at 37°C and undetectably at 4°C). It is postulated that tmICAM exists in nature in a multimeric, possibly dimeric form, and that such constructs more closely resemble the native configuration, with its attendant high avidity for the human rhinovirus. Such dimerization may conveniently be achieved in vitro by, e.g., crosslinking two ICAM monomers by chemical means or by crosslinking with appropriate antibodies, or by binding monomers to appropriate inert substrates. Multimerization can also be achieved in vivo by modification of the gene sequence coding for the select ICAM to provide appropriate binding sites in the corresponding peptide sequence. For example, muteins can be engineered which contain appropriate cysteine residues to allow in vivo multimerization via interchain disulfide bonding. Alternatively, a DNA sequence coding for an ICAM may be fused with a DNA sequence coding for an appropriate immunoglobulin or fragment thereof, such that the fusion gene product possesses at least one site suitable for interchain bonding. The resulting fusion peptide monomer can then be expressed by the cell in multimeric form. Under certain circumstances, the benefits of multimerization may also be achieved by construction of ICAM muteins containing multiple rhinovirus binding sites.

Also provided by the invention are methods for enhancing binding of ICAM and functional derivatives thereof to a ligand, i.e., human rhinovirus, and "major" group receptor viruses, lymphocyte function-associated antigen-1 (LFA-1), Plasmodium falciparum (malaria) and the like, wherein the ICAM is presented in a

multimeric configuration to the ligand to facilitate binding of the ICAM to the ligand.

The invention further comprises a method for inducing irreversible uncoating of human rhinovirus, said method comprising contacting said human rhinovirus with ICAM-1 or a fragment thereof.

This invention also provides a novel method of irreversibly inhibiting infectivity of a mammalian cell by a human rhinovirus, said method comprising contacting said human rhinovirus with ICAM-1 or a fragment thereof under conditions which allow the ICAM-1 or fragment thereof to bind to said rhinovirus; thereby stimulating irreversible uncoating of said rhinovirus.

Also provided by the invention are novel pharmaceutical compositions comprising a pharmaceutically acceptable solvent, diluent, adjuvant or carrier, and as the active ingredient, an effective amount of a polypeptide characterized by having human rhinovirus binding activity and reduction of virus infectivity. Dimeric configurations of ICAM and fragments thereof are presently preferred.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof which includes numerous illustrative examples of the practice of the invention.

DESCRIPTION OF THE FIGURES

Fig. 1 shows the protein sequences of tmICAM-1.

Fig. 2 is a schematic rendition of a) tmICAM-1, b) tICAM(453), c) tICAM(283), d) tICAM(185), and e) tICAM(88).

Fig. 3 is a schematic diagram of the constructs of Example 12: a) the heavy chain of human IgG; b) the fragment of the heavy chain used in making the immunoadhesin; c) the fragment of ICAM; d) the completed IgG/ICAM immunoadhesin.

Fig. 4 shows crosslinking of tICAM(453) into dimers by water-soluble carbodiimide/N-hydroxysuccinimide. tICAM(453) at the indicated concentrations was

crosslinked with 100 mM EDC/5 mM NHS at pH 7.5 for 18 hr at 20 C. The samples were analyzed by SDS-PAGE followed by western blotting with anti-ICAM-1 antisera. a) Western blot of crosslinked ICAM(453) showing monomer and dimer species; b) dependence of crosslinking upon tICAM(453) concentration; c) the crosslinking of tICAM(453) is not inhibited by an excess of third-party proteins.

Fig. 5 is a schematic showing construction of tICAM(1-451)/LFA-3(210-237) chimera: a) tmICAM-1; b) tICAM(1-451); c) LFA-3; d) LFA-3(210-237); e) tICAM(1-451)/ LFA-3(210-237) chimera; structure of tmICAM-1 shown for comparison.

Fig. 6 shows uncoating of HRV by tICAM(453) over 24 hours. a) shift from native 148S form to uncoated 42S form by tICAM(453); b) shift from native 148S to uncoated 42S form by tICAM(185); c)SDS-PAGE of [^{35}S]-methionine-labelled HRV-3 showing loss of VP4; d) dot-blot hybridization of RNA recovered from HRV3 species with an oligonucleotide probe for HRV. 50 ng of purified HRV3 RNA and RNA extracted from 8 ng of HRV3 species were applied to the blot.

Fig. 7 shows the predicted alignment of ICAM-1 amino acid sequence in domains IV and V onto the immunoglobulin fold motif. Arrows indicate beta strands, pointing from the N- to the C-terminus; italicized letters in bold indicate the beta strands, and numbered residues indicate cysteine residues with disulfide bonds indicated by lines. The dotted line divides the "B" and "F" faces of the domains. Residues indicated with an * are among those replaced with cysteine residues.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the following abbreviations and terms include, but are not necessarily limited to, the following definitions.

Abbreviation	Definition
ICAM	Intercellular adhesion molecule - may be used to denote both full length (trans- membrane) and truncated (non-trans- membrane) forms of the protein.
ICAM-1	Intercellular adhesion molecule-1, also known as tmICAM-1 and HRR; denoting the full-length transmembrane protein
tmICAM-1	Transmembrane intercellular adhesion molecule-1, also known as ICAM-1 and HRR; requires, e.g., detergent conditions to be solubilized
HRR	Human rhinovirus receptor, also known as ICAM-1 and tmICAM-1
sICAM-1	A naturally-occurring soluble truncated form of ICAM-1 having both the hydrophobic transmembrane domain and the carboxy-terminal cytoplasmic domain of ICAM-1 deleted; consists of amino acids 1-442 of ICAM-1 plus 11 novel amino acids; distinguishable from Staunton, et al. tICAM453 which consists of amino acids 1-453 with the terminal tyrosine replaced with phenylalanine.
tICAMs	Truncated intercellular adhesion molecules; soluble non-transmembrane ICAMs lacking the hydrophobic trans-membrane domain and the carboxyl- terminal cytoplasmic domain of ICAM-1.

tICAM(1-453)	Truncated form of ICAM comprising the entire extracellular amino-terminal domain of tmICAM (domains I - V, amino acid residues 1 - 453)
tICAM-453	
tICAM(453)	
tICAM(1-283)	Truncated form of ICAM comprising domains I, II, and III (amino acid residues 1 - 283)
tICAM-283	
tICAM(283)	
tICAM(1-185)	Truncated form of ICAM comprising domains I and II (amino acid residues 1 - 185)
tICAM-185	
tICAM(185)	
tICAM(1-88)	Truncated form of ICAM comprising domain I (amino acid residues 1 - 88)
tICAM-88	
tICAM(88)	
tICAM(89-185)	Truncated form of ICAM comprising domain II (amino acid residues 89-185)
tICAM(186-283)	Truncated form of ICAM comprising domain III (amino acid residues 186-283)
tICAM(284-385)	Truncated form of ICAM comprising domain IV (amino acid residues 284-385)
tICAM(386-453)	Truncated form of ICAM comprising domain V (amino acid residues 386-453)

tICAM(75-77)	Truncated form of ICAM comprising amino acid residues 75-77
tICAM(70-72)	Truncated form of ICAM comprising amino acid residues 70-72
tICAM(64-66)	Truncated form of ICAM comprising amino acid residues 64-66
tICAM(40-43)	Truncated form of ICAM comprising amino acid residues 40-43
tICAM(36-38)	Truncated form of ICAM comprising amino acid residues 36-38
tICAM(30-33)	Truncated form of ICAM comprising amino acid residues 30-33
tICAM(26-29)	Truncated form of ICAM comprising amino acid residues 26-29

The foregoing terms, defining specific fragments are intended to include functional derivatives and analogs thereof. Persons skilled in the art will understand that the given boundaries may vary by a few amin acid residues without affecting the function of the given fragment.

"Multimerization" and "multimeric" include, but are not limited to dimerization and dimeric, and include any multimeric configuration of the ICAM-1 molecule, or fragment thereof, that is effective in reducing viral binding and infectivity.

"Transmembrane" generally means forms of the ICAM-1 protein molecule which possess a hydrophobic membrane-spanning sequence and which are membrane-bound.

"Non-transmembrane" generally means soluble forms of the ICAM-1 protein including truncated forms of the protein that, rather than being membrane-bound, are secreted into the cell culture medium as soluble proteins, as well as transmembrane forms that have been solubilized from cell membranes by lysing cells in non-ionic detergent.

"Truncated" generally includes any protein form that is less than the full length transmembrane form of ICAM.

"Immunoadhesin" means a construct comprising all or a part of a protein or peptide fused to an immunoglobulin fragment, preferably a fragment comprising at least one constant region of an immunoglobulin heavy chain.

"Form" is generally used herein to distinguish among full length and partial length ICAM forms; whereas "configuration" is generally used to distinguish among monomeric, dimeric, and multimeric configurations of possible ICAM forms.

All forms and configurations of the ICAM-1 molecule, whether full length or a fragment thereof, including muteins and immunoadhesins, whether monomeric or multimeric, may be fully or partially glycosylated, or completely unglycosylated, as long as the molecule remains effective in reducing viral binding and infectivity.

"Ligand" is generally used herein to include anything capable of binding to at least one of any of the forms and configurations of ICAM and includes, but is not limited to, human rhinovirus, other viruses that bind to the "major" group human rhinovirus receptor, lymphocyte function-associated antigen-1, and Plasmodium falciparum (malaria).

"Human rhinovirus" generally includes all human serotypes of human rhinovirus as catalogued in Hamparian, V., et al., Virol., 159:191-192 (1987).

The sequence of amino acid residues in a peptide is designated in accordance with standard nomenclature such as that given in Lehninger's Biochemistry (Worth Publishers, New York, 1970).

Full-length ICAM-1, also known as human rhinovirus receptor (HRR), is termed transmembrane ICAM(tmICAM-1). Non-transmembrane ICAMs are also known as truncated ICAMs, i.e, ICAMs substantially without the carboxyl intracellular domain and without the hydrophobic membrane domain of tmICAM, which are soluble without the addition of detergent. tICAMs may conveniently comprise one or more domains selected substantially from domains I, II, III, IV, and V of the extracellular region of tmICAM. tICAMs may also comprise functional analogs of tmICAM or fragments thereof, and may also comprise one or more fragments of tmICAM spliced together, with or without intervening non-tmICAM lining sequences, and not necessarily in the same order found in native tmICAM. Presently preferred tICAMs include but are not limited to forms tICAM(453), tICAM(185), tICAM(88), tICAM(283), and tICAMs comprising one or more sequences selected from tICAM(89-185), tICAM(186-283), tICAM(284-385), tICAM(386-453), tICAM(75-77), tICAM(70-72), tICAM(64-66), tICAM(40-43), tICAM(36-38), tICAM(30-33), and tICAM(26-29). See USSN 07/631,313, USSN 07/678,909, and USSN 07/704,996. Non-transmembrane forms of ICAM can include functional derivatives of ICAM, mutein forms of tICAM to facilitate coupling, and tICAM immunoadhesins. When the tICAMs are in a multimeric configuration, preferably as dimers, they display enhanced binding of human rhinovirus and are able to reduce viral infectivity.

Multimerization can be achieved by crosslinking a first ICAM to a second ICAM, using suitable crosslinking agents, e.g. heterobifunctional and homobifunctional cross-linking reagents such as bifunctional N-hydroxysuccinimide esters, imidoesters, or bis-maleimidohexanes.

The different forms of ICAM, transmembrane and non-transmembrane, can be multimerized by adsorption to a support. This support can be made of materials such as nitrocellulose, PVDF, DEAE, lipid polymers, as well as amino dextran, or a variety of inert polymers that can adsorb or can be coupled to ICAM, either with or without a spacer or linker.

Multimeric ICAM can also be multimerized by coupling the ICAM to a member, e.g., an antibody that does not interfere with HRV binding, or fragments

thereof; or to a protein carrier. An example of an antibody includes anti-ICAM antibody CL 203 or a fragment thereof; suitable protein carriers include albumin and proteoglycans.

To facilitate coupling, the ICAM can be modified with at least one reactive amino acid residue such as lysine, cysteine, or other amino acid residue(s) to provide a site(s) to facilitate coupling. These types of modified ICAM are referred to as muteins. The nucleotide sequence for the ICAM of the method can be contained in a vector, such as a plasmid, and the vector can be introduced into a host cell, for example eukaryotic or prokaryotic cells. The preferred eukaryotic cell is a mammalian cell, e.g. Chinese hamster ovary cells or HEK293S cells; the preferred prokaryotic cell is E. coli. In addition, the ICAM can be modified at either terminus to comprise a lipid capable of promoting formation of oligomer micelles. The ICAM comprising the multimeric ICAM can be either fully glycosylated, partially glycosylated, or non-glycosylated.

A preferred manner of making multimeric forms of ICAM-1 is by engineering of cysteine residues into the tICAM sequence (tICAM(453) is particularly preferred) in a position at or close to the natural site of self-association on ICAM-1 monomers. Muteins with cysteine residues placed at appropriate positions form covalent bonds (disulfide bonds) that stabilize an interaction which is noncovalent in vivo. Such muteins are assembled intracellularly and are expressed as a disulfide-linked dimer; alternatively, monomeric muteins may be crosslinked in vitro by incubation at high protein concentration in mildly reducing conditions to encourage disulfide exchange, or by crosslinking with bifunctional chemical crosslinking reagents which react with free sulfhydryl groups. Another advantage of such proteins is that any novel amino acids engineered into ICAM-1 are hidden on the dimer interface and would be less likely to be immunogenic.

In another preferred embodiment, ICAM can also be multimerized by fusion with fragments of immunoglobulins to form ICAM immunoadhesins. For example, an ICAM or fragment thereof can be fused with a heavy or light chain immunoglobulin or fragment thereof, in particular with the constant region of the heavy chain of IgG, IgA, or IgM. Preferably, the constant region contains the hinge

region and one or more of CH2 and CH3, but does not contain CH1. The variable region (Fab) of the immunoglobulin is thus replaced by the ICAM or fragment thereof. Such constructs are conveniently produced by construction and expression of a suitable fusion gene in a suitable expression system [see, e.g., Bebbington, C.R. and C.C.G. Hentschel, "The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells," in DNA Cloning, Vol. III , D. Glover, ed.(1987)] and are secreted in a dimerized configuration.

Also provided by the invention are methods for enhancing binding of ICAM and functional derivatives thereof to a ligand, i.e., human rhinovirus, and "major" group receptor viruses, lymphocyte function-associated antigen-l (LFA-l), Plasmodium falciparum (malaria) and the like, wherein the ICAM is presented in a multimeric configuration to the ligand to facilitate binding of the ICAM to the ligand.

The invention further comprises a method for inducing irreversible uncoating of human rhinovirus, said method comprising contacting said human rhinovirus with ICAM-1 or a fragment thereof, e.g. a tICAM as defined above.

This invention also provides a novel method of irreversibly inhibiting infectivity of a mammalian cell by a human rhinovirus, said method comprising contacting said human rhinovirus with ICAM-1 or a fragment thereof under conditions which allow the ICAM-1 or fragment thereof (e.g. a tICAM as defined above) to bind to said rhinovirus; thereby stimulating irreversible uncoating of said rhinovirus.

Also provided by the invention are novel pharmaceutical compositions comprising a pharmaceutically acceptable solvent, diluent, adjuvant or carrier, and as the active ingredient, an effective amount of a polypeptide characterized by having human rhinovirus binding activity and reduction of virus infectivity. Dimeric configurations of ICAM and fragments thereof are presently preferred.

The following examples illustrate practice of the invention.

Example 1 relates to growth, purification and assay of rhinoviruses;

Example 2 relates to production and isolation of monoclonal antibodies to ICAM-1;

Example 3 relates to construction of non-transmembrane truncated forms of ICAM cDNA from full length ICAM-1 cDNA;

Example 4 relates to transfection of mammalian-cells and expression of non-transmembrane truncated forms of ICAM cDNA;

Example 5 relates to isolation and purification of non-transmembrane truncated forms of ICAM-1;

Example 6 relates to radioactive labeling of tmICAM-1, tICAM(185), and tICAM(453) and demonstration of retained capacity for binding to monoclonal antibodies;

Example 7 relates to human rhinovirus binding assays of transmembrane and of non-transmembrane truncated forms of ICAM-1;

Example 8 relates to CL203 IgG antibody-mediated cross-linking of tICAM(453);

Example 9 relates to multimerization of trans-membrane and of non-transmembrane truncated forms of ICAM-1;

Example 10 relates to infectivity-neutralization assay of multimeric transmembrane and of multimeric non-transmembrane truncated forms of ICAM-1; and

Example 11 relates to use of multimeric forms of transmembrane and truncated forms of ICAM-1, as effective inhibitors of ICAM/LFA-1 interaction.

Example 12 relates to construction of tICAM(185)/IgG and tICAM(453)/IgG immunoadhesins.

Example 13 relates to rhinovirus binding and neutralization by a tICAM/IgG immunoadhesins.

Example 14 relates to in vitro dimerization of ICAM-1.

Example 15 relates to a tICAM(1-451)/LFA-3(210-237) chimera.

Example 16 relates to irreversible inactivation of HRV by ICAM.

Example 17 relates to cysteine muteins.

EXAMPLE 1

GROWTH, PURIFICATION AND ASSAY OF RHINOVIRUSES

Rhinoviruses were grown, purified, and assayed essentially as described in Abraham, G., et al., J. Virol., 51:340 (1984) and Greve, et al., Cell, 56:839 (1989). The serotypes chosen for these studies include HRV14, the standard in the field, and HRV3, which has an approximately 10-fold higher affinity for ICAM than does HRV14. HRV2, which binds to the "minor" receptor rather than the "major" receptor, was used as a negative control.

Rhinoviruses HRV2, HRV3, and HRV14 were obtained from the American Type Culture Collection, plaque purified, and isolated from lysates of infected HeLa-S3 cells. Purified rhinovirus was prepared by polyethylene glycol precipitation and sucrose gradient sedimentation. Viral purity was assessed by SDS-PAGE analysis of capsid proteins and by electron microscopy. Infectivity was quantitated by a limiting dilution infectivity assay scoring for cytopathic effect, essentially as described by Minor, P.D., Growth, assay and purification of picornaviruses, in Virology:A Practical Approach, B.W.J. Mahy, ed (Oxford:IRL Press), pp. 25-41.

EXAMPLE 2

PRODUCTION AND ISOLATION OF MONOCLONAL ANTIBODIES TO ICAM-1

BALB/cByJ female mice were immunized by intraperitoneal injection of 107 intact HeLa cells in 0.5 ml of phosphate-buffered saline (PBS) three times at 3-week intervals. Two weeks later the mice were bled and aliquots of serum were tested for protective effects against HRV14 infection of HeLa cells. Positive mice were boosted by a final injection of 10^7 HeLa cells, and 3 days later spleen cells were fused to P3X63-Ag8.653 myeloma cells (Galfre, et al., Nature, 266:550-552 (1977)) to produce a total of approximately 700 hybridoma-containing wells. Each well was tested by incubating 3 x 10^4 HeLa cells in 96-well plates with 100 μl of supernatant for 1 hr at 37 C; the cells were then washed with PBS, and a sufficient amount of HRV14 was added to give complete cytopathic effect in 24-36 hr. Wells that were positive (protected from infection) were scored at 36 hr.

Cells were removed from wells which scored positive in the first screen and cloned by limiting dilution in 96-well microtiter plates. Supernatants from these wells were tested in the cell protection assay and positive wells were again identified. Further clonings were performed until all of the hybridoma containing wells were positive indicating a clonal population had been obtained. Four cloned cell lines, and their corresponding antibodies, were obtained and were designated c78.1A, c78.2A, c78.4A, c78.5A, c92.1A and c92.5A, respectively.

C92.1A was deposited on November 19, 1987 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 and was designated HB 9594.

EXAMPLE 3

CONSTRUCTION OF tICAM cDNAs FROM FULL LENGTH ICAM-1 cDNA

A. Preparation of ICAM-1 cDNA

Randomly-primed cDNA was synthesized from poly A+ RNA from HE1 cells using an Amersham(TM) cDNA synthesis kit under conditions recommended by the supplier. PCR amplification was performed using 100 ng of cDNA for 25 cycles using primers PCR 5.1: (ggaattcATGGCTCCCAGCAGCCCCCGGCCC) and PCR 3.1: (ggaattcTCAGGGAGGCGTGGCTTGTGTGTT). Amplification cycles consisted of 94 C 1 min, 55 C 2 min, and 72 C 4 min. The product of the PCR reaction was digested with EcoR1 and cloned with EcoR1 digested phage vector lambdaGT10 (Stratagene(TM)). Recombinant phage clones were screened by plaque hybridization using ICAM-1 specific oligonucleotides

GAGGTGTTCTCAAACAGCTCCAGCCCTTGGGGCCGCAGGTCCAGTTC (ICAM1) and

CGCTGGCAGGACAAAGGTCTGGAGCTGGTAGGGGGCCGAGGTGTTCT (ICAM3).

A positive clone designated lambdaHRR4 was selected and purified. The insert was removed by EcoR1 digestion and subcloned into the EcoR1 site of Bluescript KS+. This clone was designated pHRR2. The entire insert was

sequenced and found to contain the entire ICAM-1 coding sequence beginning with the initiator ATG codon and ending with the TGA stop codon as specified by the PCR ICAM-1 sequence (Simmons, et al., Nature, 331:624 (1988); Staunton, et al., Cell, 52:925-933 (1988)) by a single substitution of Ala-1462 for Gly. This same change was identified in several independent clones and thus represents a polymorphism of the ICAM-1 gene.

B. Construction of tICAM(453) and tICAM(185)

Modified forms of the ICAM-1 cDNA were created by PCR amplification reactions (Saiki, et al., Science, 230:1350-1354 (1985)) using the full length ICAM-1 cDNA clone pHRR-2 as template. The plasmid DNA was digested with EcoR1 to excise the ICAM-1 insert and treated with alkaline phosphatase to prevent re-circularization of the vector in subsequent ligation steps. Ten ng of template DNA was subjected to 10 cycles of PCR amplification using oligonucleotide primers PCR5.5 and PCR3.3 for tICAM-453 and PCR5.5 and 3.10 for tICAM-185 under the following conditions:

Temperature (°C)	Time (mins)
94	1
55	2
72	1.5
71	4 (final extension)

PCR5.5 has the sequence: GGAATTCAAGCTTCTCAGCCTCGCTATGG-CTCCCAGCAGCCCCCGGCCC which consists of EcoR1 and HindIII sites, 12bp ICAM-1 5' untranslated sequence, and the first 24 bp encoding the signal peptide.

PCR3.3 has the sequence: GGAATTCCTGCAGTCACTCATACCGGGGG-GAGAGCACATT which consists of EcoR1 and Pst1 sites, a stop codon, and 24 bp complementary to the bases encoding the last 8 extracellular amino acids of ICAM-1 (residues 446-453).

PCR3.10 has the sequence: TTCTAGAGGATCCTCAAAAGGTCTGGAG-CTGGTAGGGGG which consists of Xba1 and BamH1 sites, a stop codon, and 24 bp complementary to the bases encoding residues 178-185 of ICAM-1.

The PCR reaction products were digested with EcoR1 (tICAM(453)) or EcoR1 and BamH1 (tICAM(185)) and cloned into the polylinker site of Bluescript SK+ (Stratagene). Clones containing the desired inserts were verified by restriction analysis and DNA sequencing. The inserts were excised from Bluescript by digestion with HindIII and XbaI and inserted into the expression vector CDM8 (Seed, Nature, 239:840 (1987) at the HindIII and XbaI sites. A clone containing the tICAM(453) insert designated pHRR-8.2 and a clone containing the tICAM(185) insert designated pHRR23-13 were selected and subjected to extensive sequence analysis. This verified the existence of the desired stop codons, and the integrity of the selected regions of ICAM-1 coding sequence.

These plasmids were transfected into COS cells using the DEAE-dextran techniques and the cells were cultured 72 hr. before assay. Surface expression was monitored by FACS using indirect immunofluorescence and a monoclonal antibody specific for ICAM-1. Transient expression in COS cells and immunoprecipitation of metabolically labelled ([^{35}S]cysteine) cell supernatants with c78.4A Mab (monoclonal antibody) demonstrated the production of soluble ICAM-1 fragments of 45 kd and 80 kd from pHRR23-13 and pHRR8.2, respectively. The preparation of stable Chinese hamster ovary cell transfectants is described below in Example 4.

C. Modified Non-glycosylated tICAM-1

A modified full length ICAM-1 was made by simultaneous mutagenesis of Asn at positions 103, 118, 156 and 173 each to Gln. This removes all four Asn-linked glycosylation sites from extracellular domain II of the ICAM-1 molecule. The resultant molecule, referred to as non-glycosylated transmembrane ICAM, was expressed on the surface of COS cells and was able to bind radio-labeled HRV3 at levels comparable to unmodified ICAM-1. This result demonstrated that glycosylation of domain II (the first 185 amino acids) is not required for virus binding to ICAM-1.

It is expected that non-transmembrane ICAM can be similarly modified to yield modified non-glycosylated non-transmembrane ICAM-1 molecules.

D. Construction of Genetically Engineered Forms of tICAM Containing Reactive Residues Suitable for Cross-Linking to Form Multimers

A molecule consisting of the 453 amino acid extracellular domain of ICAM-1 with the addition of a novel lysine residue at the C-terminus was constructed by PCR modification of the pHRR-2 cDNA described in Example 3B. The primers used were PCR5.5 (Example 3B) and PCR 3.19 which has the sequence: TTCTAGAGGATCCTCACTTCTCATACCGGGGGGAGAGCACATT and consists of XbaI and BamHI sites, a stop codon, a Lys codon, and 24 bases complementary to the sequence encoding amino acid residues 446 to 453. Following cloning into the CDM8 vector, production of tICAM having a Lys at position 453 was confirmed by transient expression in COS cells. Stable CHO cell lines were generated by co-transfection with pSV2-DHFR as described in Example 4. The same strategy was used to add a Lys residue to the C-terminus of tICAM(185) using PCR5.5 and PCR3.20 which has the sequence: TTCTAGAGGATCCTCACTTAAAGGTCTGGAGCTGGTAGGGGGC and consists of XbaI and BamHI sites, a stop codon, a Lys codon, and 24 bases complementary to the sequence encoding residues 178 to 185. Transient COS cell expression confirmed the production of tICAM-185 and stable CHO cell lines were derived as described in Example 4.

Three modified forms of tICAM(452) that each contain an additional Cys residue were constructed by site-directed mutagenesis of the full-length ICAM-1 cDNA. In each construct a stop codon was introduced by changing the Glu residue at position 453 from GAG to TAG. The C-terminus is thus Tyr-452. Residues Asn-338, Thr-360, and Gln-387 were each separately mutated to Cys using a second site directed mutagenesis. The presence of the desired mutations were confirmed by DNA sequencing.

The residues selected for mutation to Cys were selected based on a computer generated plot of surface probability which predicts surface exposure of these regions.

Also, Thr-360 is in close proximity to Asn-358 which is a site of potential Asn-linked glycosylation. Each of the three Cys mutants was expressed and secreted into the medium of transfected COS cells. Examination of the proteins under reducing and non-reducing conditions showed no indication of the presence of dimers. It is anticipated that cross-linking reagents reactive with sulfhydryl groups can be used to cross-link the Cys-modified tICAM forms to obtain multimeric forms.

EXAMPLE 4

TRANSFECTION OF CELLS AND EXPRESSION OF tICAM cDNA

A. Transfection of Eukaryotic Cells

Chinese hamster ovary (CHO) cells deficient in dihydrofolate reductase (DHFR) were obtained from Cutter Labs (Berkeley, CA.). DHFR- cells cannot synthesize nucleosides and therefore require a nucleoside-supplemented medium. The cells were co-transfected with the plasmid pSV2-DHFR which contains the mouse dihydrofolate reductase (DHFR) gene under control of the SV40 promoter, and with tICAM(453), or tICAM(184) constructs in the CDM8 vector (Seed and Aruffo, PNAS, 84:3365-3369 (1987)).

Transfections were done using both electroporation and calcium phosphate methods. Bebbington, supra. Transfected DHFR-positive cells were selected by growth on nucleoside-free media, and pools of transfectants were cloned by limiting dilution.

Cell lines that secrete tICAM were identified by testing culture supernatants with a two-site radioimmune assay (RIA) for ICAM using Mabs c78.4A and c78.5A as follows. A monoclonal antibody against one epitope on ICAM (for example, Mab c78.4A) was adsorbed to plastic 96-well plates (Immunlon plates, Dynatech Inc.), excess binding sites on the plates were blocked with bovine serum albumin (BSA), and then culture supernatants were incubated with the plates. The plates were washed and incubated with [^{125}I]-Mab (directed against a second epitope on ICAM, e.g. c78.5A), and, after washing, the amount of bound [^{125}]I-IgG determined. The concentration of tICAM was determined by comparing RIA data from unknowns

against a standard curve of tmICAM at known concentrations. Positive clones were expanded and expression of tICAM forms was confirmed by immunoprecipitation of metabolically labeled cell supernatants with Mab c78.4A.

Cell lines CT.2A (tICAM(453)) and CD12.1A (tICAM(185)) were selected for further study and were subjected to gene amplification in methotrexate containing media as described by Bebbington, et al., supra. A clone derived from CT.2A resistant to 100 nM methotrexate and a CD12.1A clone resistant to 1 μM methotrexate were used for purification of soluble truncated ICAM-1 proteins.

B. Transfection of Prokaryotic Cells

Because glycosylation of the viral binding domain of ICAM is not required to retain viral binding (as demonstrated in Example 3C), it is anticipated that prokaryotic cells, such as E. coli, can be successfully transfected to produce functional proteins.

EXAMPLE 5

ISOLATION AND PURIFICATION OF tICAM-1

Monoclonal antibody affinity chromatography with c78.4A-Sepharose(TM) has been previously described in co-pending USSN 07/130,378 and Greve, et al., Cell, 56:839-847 (1989). tICAM secreted into serum-containing media required additional purification steps due to the high level of contaminating protein in the serum. Before elution from the Mab-affinity column, the column was washed with 1 M NaCl to remove loosely-bound proteins. For tICAM(453), the partially purified tICAM(453) eluted from the c78.4-Sepharose(TM) column was dialyzed into 10 mM Tris (pH 6.0), absorbed onto a mono-Q(TM) column (Pharmacia), and eluted with a 0-0.3 M NaCl gradient. tICAM184 was further purified by gel filtration on a Superose-12(TM) column.

It is also recognized that non-transmembrane truncated forms of ICAM-1 may be purified using standard ion exchange methodology without using monoclonal antibody affinity chromatography.

EXAMPLE 6

RADIOACTIVE LABELING OF tmICAM-1, tICAM(185), AND tICAM(453) AND DEMONSTRATION OF RETAINED CAPACITY FOR BINDING TO MONOCLONAL ANTIBODIES

The epitopes reactive with monoclonal antibodies c78.4A and c78.5A are conformationally-dependent epitopes and thus can be used as analytical probes for confirming retention of the native ICAM structure. Known amounts of purified ICAM were incubated with c78.4A or c78.5A IgG-Sepharose(TM) and the fraction of the radioactivity bound determined. These experiments showed that the purified tmICAM-1, tICAM(185), and tICAM(453) completely retained the ability to bind to these monoclonal antibodies.

Transfectants were metabolically labeled with [^{35}S]cysteine, and cell lysates (for transmembrane ICAM) or culture supernatants (for truncated ICAM) were prepared and incubated with c78.4A IgG-Sepharose(TM) beads. The beads were washed and adsorbed proteins were eluted with sodium dodecyl sulfate (SDS) and analysed by SDS-PAGE; see Greve, et al., Cell, 56:839-847 (1989)). It was found that the isolated proteins were quantitatively bound to the c78.4A and c78.5A Mabs.

Accordingly, the tICAM(185) and tICAM(453) both have retained native ICAM structure.

EXAMPLE 7

HUMAN RHINOVIRUS BINDING ASSAYS OF tmICAM AND tICAMs

Described below are three binding assays used to assess binding activity of the various forms of ICAM.

A. Pelleting Assay

[^{35}S]cysteine-labeled tmICAM-1 or tICAM was mixed with HRV3 in 100 μl of 10 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% Triton X-100. The mixture was incubated for 30 min. at 37 C, cooled on ice, layered on top of a cushion of 200 μl of 10% glycerol, 0.2 M triethanolamine (pH 7.5), and centrifuged in a Beckman air-driven centrifuge at 134,000 x g for 30 min. at 4 C.

The top 275 μl was removed, and the pellet was analyzed by SDS-PAGE and scintillation counting. Silver-staining of SDS gels of control experiments indicated that essentially all of the HRV3 is pelleted under these conditions and essentially all of the ICAM remains in the supernatant. The results are shown in Table 1.

TABLE 1

ICAM	% ICAM Pelleted*
tmICAM-1	11.6%
tICAM(453)	1.0%
tICAM(185)	4.3%

* average of 4 experiments; these numbers cannot be directly converted into relative affinities

These data show that both truncated forms of ICAM bind to rhinovirus, but at substantially reduced levels relative to tmICAM.

B. Solution Binding Assay

To obtain quantitative information on the relative affinity of tmICAM and tICAM fragments in solution, a solution competition assay was developed in which soluble tmICAM or soluble tICAM fragments were used to inhibit the binding of [^{35}S]HRV3 to previously immobilized ICAM-1; nonionic detergent (Triton X-100) was included in the buffers so that the different proteins could be compared under identical conditions. First, tmICAM-1 (isolated in the presence of 0.1% octylglucoside instead of Triton X-100) was diluted 10-fold into a Tris/NaCl buffer and allowed to adsorb to the walls of a microtiter plate (Immunlon-4, Dynatech) overnight. Nonspecific binding sites on the plate were then blocked with 10 mg/ml BSA and binding experiments performed in 0.1% Triton X-100/1 mg/ml BSA/10 mM Tris/200 mM NaCl. Approximately 20,000 cpm of [^{35}S]HRV3 were mixed with varying amounts of ICAM [tmICAM, tICAM(453) or tICAM(185)], incubated for 1

hour at 37 C, and then added to wells of the microtiter plates and incubated for 3 hr at 37 C. The plates were washed and the bound radioactivity determined.

As shown in Table 2, tmICAM-1 inhibits virus binding half-maximally at low concentrations (.008 μM) while tICAM(453) and tICAM(185) inhibit at much higher concentrations (2.8 μM and 7.9 μM, respectively; or 350 to almost 1000-fold higher than tmICAM.

TABLE 2

ICAM	IC50*
tmICAM	8.0 $\pm$ 3.3 nM (N=3)
tICAM(453)	2.8 $\pm$ 0.6 μM (N=3)
tICAM(185)	7.9 $\pm$ 2.8 μM (N=3)

* IC50 is the concentration of soluble ICAM needed to inhibit HRV3 binding by 50%.

These data confirm and extend the earlier observations that tICAM(453) and tICAM(185) do bind to rhinovirus but with lower affinities than does tmICAM-1 and provide evidence that the virus binding site is encompassed within the two N-terminal domains (185 residues) of ICAM-1.

Subsequent experiments performed at 34 C (the temperature at which rhinovirus normally replicates) have yielded similar results.

C. Dot-Blot Assay

An alternative method of measuring binding activity was utilized in which tmICAM-1, tICAM(453), or tICAM(185) was adsorbed to nitrocellulose filters, the non-specific binding sites on the filters blocked with 10 mg/ml bovine serum albumin (BSA), and radioactive virus or [^{125}I]Mab to ICAM-1 incubated with the filter for 60 min at 37 C. The filters were washed with buffer and the filters exposed to X-ray film.

The amount of radioactivity bound to the filters was determined by densitometry of the autoradiograms, and the data is expressed as HRV3 binding (in arbitrary units) normalized to the amount of ICAM bound to the blot by a parallel determination of the amount of [^{125}I]Mab c78.4A or c78.5A bound to the ICAM (bound to the blot). The results are shown in Table 3.

TABLE 3

Binding of [^{35}S]HRV3 to Immobilized ICAM*

ICAM	tICAM(453)	ratio ICAM/tICAM453
1.2 ± 1.1	0.52 ± 0.45	2.3

* Average of 5 experiments. Data is expressed in arbitrary densitometric units of [^{35}S]HRV3 binding/[^{125}]I anti-ICAM Mab binding.

Additional studies with tICAM 185 have been performed. Binding experiments have demonstrated equivocal results. It is anticipated that steric hindrance may play a role. The size of the virus is approximately 30 nanometers. The length of tICAM(185) is less than 10 nanometers. The use of a spacer or linker would provide better accessibility for binding.

The results from this experiment indicate that under these assay conditions tICAM(453) is capable of binding rhinovirus at levels comparable to those of tmICAM-1 when the amount of virus bound was normalized to the amount of [^{125}I]MAb bound. Further, these results indicate that the tICAM forms are capable of binding to rhinovirus, but that the binding avidity is dependent upon the configuration of the tICAM. tmICAM-1 is believed to be a small multimer (probably a dimer) and presentation of tICAM in a multimeric form mimics this multimeric configuration.

Evidence supporting this hypothesis comes from quantitative binding studies (unpublished), in which the ratio of the maximum number of rhinovirus particles and the maximum number of antibody molecules that can be bound to cells is approximately 1.5, as discussed supra. This is in contrast to the earlier work of Tomassini, J., et al., J. Virol., 58:290 (1986), which suggested a complex of five

molecules needed for binding. Their conclusion was based on an erroneous interpretation of gel filtration data that failed to take into account bound detergent molecules.

EXAMPLE 8

CL203 IgG ANTIBODY-MEDIATED CROSS-LINKING OF tICAM(453)

To provide additional evidence that the higher relative binding activity of tmICAM-1 is due to a multimeric form of the protein, the tICAM(453) protein was pre-incubated with CL203, a monoclonal antibody to ICAM-1 that does not inhibit virus binding to ICAM-1 and binds to a site C-terminal to residue 184 (Staunton, et al., Cell, 56:849 (1989) and Cell, 61:243 (1990)). Thus, the antibody can effectively "cross-link" two molecules of tICAM(453), to create "dimers" of tICAM(453), yet without blocking the virus-binding site on each of the two molecules of tICAM(453). When a mixture of CL203 IgG and tICAM(453) at a 4:1 weight ratio was tested in the competition assay, it was found that the antibody cross-linked tICAM(453) inhibited HRV3 binding at a concentration 7.4-fold lower than tICAM(453) alone consistent with the idea that tmICAM-1 binds with higher affinity to rhinovirus because it is a dimer or a small multimer.

To create alternative multimeric forms of tICAM, several further modified truncated forms of ICAM were constructed as described, supra, in Example 3.

These forms can then be multimerized as described in Example 9, below.

EXAMPLE 9

MULTIMERIZATION OF tmICAM AND tICAMs

There are several ways that tICAM can be converted to a multimeric form having enhanced viral binding and neutralization activity over the monomeric form. For example, a first tICAM can be coupled to a second tICAM(which may be the same or different), or to an inert polymer, such as amino-dextran (MW 40,000), using homobifunctional (such as N-hydroxysuccinimide (NHS) esters) or heterobifunctional (such as those containing NHS-ester and photoactivatable or

sulfhydryl-reactive groups) cross-linking reagents utilizing the amino group on the amino-dextran and an amino or other group on the tICAM. A number of examples of appropriate cross-linking reagents can be found in the Pierce Chemical Company catalog (Rockford, Ill.). Similarly, the tICAMs can also be bound to other suitable inert polymers, such as nitrocellulose, PVDF, DEAE, lipid polymer, and other inert polymers that can adsorb or be coupled to tICAM with or without a spacer or linker.

As tICAM is poorly reactive with NHS-ester-based compounds, a tICAM with a genetically-engineered C-terminal lysine residue (see Example 3) would have improved coupling efficiency to supports with homobifunctional reagents whereas genetically-engineered C-terminal cysteine residues would facilitate coupling by heterobifunctional reagents, such as sulfo-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS).

ICAMs can also be multimerized by coupling with an antibody (e.g. CL203) or fragment thereof, or with a suitable protein carrier, e.g. albumin or proteoglycan.

ICAMs may also be multimerized by fusion with fragments of immunoglobulins to form ICAM immunoadhesins.

Alternatively, soluble tICAM multimers can be created by genetically engineering reactive residues into tICAM. For example, free cysteine residues can be created in relatively hydrophilic sequences in the C-terminal region of tICAM (which would have a greater tendency to be solvent-exposed). This will allow the creation of dimers in situ; alternatively, monomers can be purified and dimers created in vitro by disulfide bonding, either directly or via suitable linkers.

Another approach requires the placement of lysine residues at similar positions and cross-linking purified protein in vitro with homobifunctional NHS-esters. Examples of such lysine residues are residues 338, 360, 387. See Fig. 1.

Crosslinking cysteine residues to each other can be accomplished by reaction of tICAM with free cysteine groups with bis-maleimidohexane (Pierce Chemical Co.) or other bis-maleimido-analogs. Cross-linking free cysteine residues on tICAM to amino groups on carrier molecules can be accomplished by reaction with m-maleimidobenzoyl-N-hydroxy- succinimide ester.

Crosslinking amino groups on tICAM molecules can be accomplished with homobifunctional N-hydroxysuccinimide esters (for examples, see Pierce Chemical Co. catalog). Alternatively, the carbohydrate groups on tICAM can be oxidized to aldehydes and coupled to hydrazine-activated amino groups on a carrier molecule.

EXAMPLE 10

INFECTIVITY-NEUTRALIZATION ASSAY OF tmICAM AND tICAMs

Three different assays for virus infectivity have been used. These different assays take into account the differences in transmembrane ICAM and non-transmembrane solubilities.

A. Plaque-reduction assay in the presence of detergent

The results of this assay indicate the highest dilution of virus that will still be effective in killing cells. Virus is pre-incubated with transmembrane ICAM protein in the presence of 0.1% Triton X100, serially diluted into culture medium, incubated for 30 min with HeLa cells at 10^6 cells/ml, diluted 10-fold, and plated out into multiple wells of a 96-well microtiter plate having varying dilutions of virus.

0.1% Triton X100 was used as positive control. After 5 days, the wells are scored as either being infected or not by the presence of cytopathic effect (CPE) and the titer expressed as plaque-forming units/ml (PFU/ml) of the original virus. This assay was described in USSN 07/239,571 and was used to demonstrate the antiviral activity of tmICAM-1 (which required the presence of detergent to remain in solution). The concentration of ICAM protein used is the initial concentration in the pre-incubation mixture; however, the ICAM protein is not present continually during the infection in that the protein is serially diluted. While the presence of detergent is required to solubilize the tmICAM, detergent kills the cells; thus, the need for the serial dilutions of the tmICAM-1/detergent to permit infection of cells.

B. Plaque-reduction assay in the absence of detergent

In this plaque-reduction assay, a more traditional assay, HeLa cells are infected with serial dilution of rhinovirus as above, but detergent is not present; thus,

this assay cannot be used to assay tmICAM. In this assay the tICAM is present continually in the culture medium at the indicated concentration. tmICAM-1 (which requires the presence of detergent) cannot be assayed in this system because the addition of the required detergent would kill the HeLa cells.

C. Plaque-reduction assay in continual presence of virus and ICAM

This assay is similar to that utilized by Marlin, et al. (Nature 1990) in which a culture of HeLa cells is infected with 100 PFU of virus in the presence or absence of ICAM protein and cultured approximately 4 days until cytopathic effect (CPE) is apparent. The cultures are then scored for CPE visually. The assay conditions were the same as Marlin, supra. Scoring was done visually rather than by a staining procedure using crystal violet.

In this assay, there is no detergent present, the ICAM is present continually, and this assay measures a reduction in virus replication/propagation at an arbitrary point in time.

The data from these three different assays for virus infectivity is summarized in Table 4.

TABLE 4

	IC50% (μM)*		
ICAM	Assay: A	B	C
tmICAM-1	0.03	ND	
tICAM(453)	>20	0.2	0.2
tICAM(185)	>20	8	ND

* IC50% is defined as the concentration of ICAM protein needed to inhibit HRV3 infectivity by 50%.

These data indicate that tmICAM-1 is significantly more active in reducing viral infectivity than the truncated ICAM proteins, even when compared in different assay systems. The differences in neutralization activity of tICAM(453) in assay (A)

and assay (B) indicate that the neutralization mediated by tICAM(453) requires the continual presence of tICAM(453) in the culture medium and is reversible. That the neutralization is reversible is indicated by the lack of significant neutralization observed in assay (A). In contrast, the neutralization activity of tmICAM-1 is >667-fold higher than tICAM(453) and than tICAM(185) in assay (A) and could be even greater in assay (B) if it were possible to have the tmICAM-1 present continually in the culture medium in the absence of detergent. The conditions in assays B-D more closely reflect the in vivo situation in which soluble ICAM could be used as an antiviral agent.

To compare these results with those of Marlin et al., an attempt was made to reproduce their assay conditions. As shown in Table 4, there is a good correlation between the results in assay (B) and assay (C), although the IC50% for tICAM(453) is 10-fold greater than that seen by Marlin et al. To determine if this is due to a difference in the serotype of rhinovirus used, the assay was repeated with HRV14 and HRV54 (the serotype used by Marlin, et al.). The IC50% for both of these serotypes was 0.2 μM tICAM(453), indicating that there is no difference in serotype sensitivity between HRV14, HRV54, and HRV3.

To attempt to resolve this discrepancy, the same buffers that Marlin, et al. used were used to see if they affected the infectivity of rhinovirus in assay (C). Marlin, et al. prepared their sICAM-1 protein in a buffer containing 50 mM triethanolamine (TEA)/20 mM Tris. When this buffer alone was added to control infections (1/10th volume, final concentration 5 mM TEA/2 mM Tris) of HRV3 and HRV14, virtually complete inhibition of CPE was observed. Thus, it is possible that there could be buffer effects on virus replication unrelated to the presence of any form of ICAM.

However, subsequent assays using a broad panel of HRV serotypes indicates that the IC50% for HRV54 may in fact be significantly lower than for other HRV serotypes, e.g. HRV3.

EXAMPLE 11

USE OF MULTIMERIC FORMS OF tmICAM AND tICAMs AS EFFECTIVE INHIBITORS OF ICAM/LFA-1 INTERACTION

The normal function of ICAM-1 is to serve as a ligand of the leukocyte integrin LFA-1; interaction between these two molecules leads to adhesion between leukocytes and a variety of other cells. The ability of tICAMs to inhibit adhesion between ICAM-1 and LFA-1 on cells was examined as follows. ICAM-1 was adsorbed to microtiter plates as described in Example 7C. JY cells, which express LFA-1, adhere to ICAM-expressing cells or to ICAM-1-coated culture dishes (Staunton, et al., JCB). JY cells (10^7 cell/ml in 10 mM HEPES pH 7.5/150 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$ containing 1 mg/ml BSA) labeled with 10 μCi/ml [^{35}S]-cysteine for 18 hours) were pre-incubated in the presence or absence of tICAM(453) or tICAM(185) for 30 min at 37 C, and then added to the ICAM-1-coated plates and incubated for 60 min at 37 C. The microtiter plates were then washed three times with media, and the number of cells bound to the plates were quantified by scintillation counting.

As shown in Table 5, tICAM(185) and tICAM(453) both inhibited JY cell binding at identical concentrations of between 5 and 20 μM.

TABLE 5

	% JY Cell Binding	
μM ICAM-1	tICAM(453)	tICAM(185)
20	100	
6	5	
2	47	50
0.6	83	72
0.02	86	80
0.006	89	97

*Binding to ICAM-1-coated microtiter plates; 10 μg/ml anti-LFA-1 or anti-ICAM-1 MAb inhibited binding to <1%.

EXAMPLE 12

Construction of tICAM/IgG Immunoadhesins

A soluble derivative of ICAM-1 was constructed by a cDNA fusion which linked the first two domains of ICAM-1 (residues 1-185) to a segment of human immunoglobulin heavy chain cDNA. This approach has been described previously for the CD4 molecule [Zettlmeissl, G., J-P Gregersen, J.M. Duport, S. Mehdi, G. Reiner, and B. Seed, "Expression and Characterization of Human CD4: Immunoglobulin Fusion Proteins", DNA and Cell Biology (1990) 9(5):347-353; Capon, D.J., S.M. Chamow, J. Mordenti, S.A. Marsters, T. Gregory, H. Mitsuya, R.A. Bryn, C. Lucas, F.M. Wurm, J.E. Groopman, S. Broder, and D.H. Smith, "Designing CD4 immunoadhesins for AIDS therapy", Nature (1989) 337:525-531; Traunecker, A. J. Schneider, H. Kiefer and K. Karjalainen, "Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules", Nature (1989) 339:68-70] and resulted in the expression of disulfide-linked dimers.

The cDNA fusion was accomplished by a two-stage polymerase chain reaction (PCR) strategy. [See, e.g., Horton, R.M., Z. Cai, S.N. Ho, and L.R. Pease, "Gene Splicing by Overlap Extension: Tailor-Made Genes Using the Polymerase Chain Reaction", BioTechniques (1990) 8(5):528-535]. The first step involved the separate amplification of a fragment coding for residues 1-185 of ICAM-1 and an IgG heavy chain fragment beginning at residue 216 in the hinge region and ending at the C-terminus of the molecule (see Fig. 3). The PCR primer used at the 3' end of the ICAM-1 fragment contained an additional 24 bases complementary to the first 24 bases of the IgG fragment: CGG TGG GCA TGT GTG AGT TTT GTC AAA GGT CTG GAG CTG GTA GGG GGC. The 5' ICAM-1 primer (5' noncoding and signal sequence) had the sequence:

```
                HindIII
GGA ATT CAA GCT TCT CAG CCT CGC TAT GGC TCC CAG CAG CCC CCG GCC C
```

The 5' IgG primer had the following sequence: GAC AAA ACT CAC ACA TGC CCA CGG; the 3' primer from the end of the IgG coding sequence was:

XbaI
G GGA TTC TCT AGA TCA TTT ACC CGG AGA CAG GGA GAG GCT

Amplifications were performed using 10 ng of cloned ICAM-1 or IgG1 heavy chain cDNA for 10 cycles with 1 min at 94 C, 2 min at 55 C and 1.5 min extensions at 72 C. The resulting amplified fragments were mixed in approximately equimolar amounts and used as template for the second step PCR reaction. This reaction used the 5' ICAM primer and the 3' IgG primer above. Amplification for 25 cycles under the same conditions as in the first step produced a predominant band of approximately 1200 bp consistent with the desired product (see Fig. 3). The fragment was digested with HindIII and XbaI (restriction sites incorporated into the 5' and 3' primers respectively), purified and ligated into HindIII/XbaI-cleaved CDM8 vector.

Clones containing the desired insert were identified by restriction analysis and two clones designated pHRR72 and pHRR73 were selected for sequence analysis. Sequencing of the junction region between ICAM-1 and the IgG hinge confirmed that both clones had the correct structure. The plasmids were transfected into COS cells which were labelled with [^{35}S]cysteine overnight at 48 hours post-transfection as in Example 6. The supernatants were immunoprecipitated with anti-ICAM-1 monoclonal antibody c78.4A and analyzed by SDS gel electrophoresis as in Example 6. Under reducing conditions a band with an apparent molecular weight of 68 kD was specifically immunoprecipitated, corresponding to the ICAM-1/IgG fusion. Expression of clone pHRR72 was consistently higher than pHRR73 so this clone was selected for further study.

COS cells were transfected with pHRR72 according to the method of Example 3 and at 48 hours after transfection the media was replaced with serum-free media containing [^{35}S]cysteine and the cells were labelled overnight as above. The supernatants were incubated with protein A- Sepharose beads, and bound protein was eluted with 0.1 M acetic acid, neutralized and analyzed by gel electrophoresis under reducing and non-reducing conditions. A control was performed in which plasmids expressing heavy and light chains of a functional antibody were co-transfected. This experiment showed that the protein produced by pHRR72 is capable of binding protein A, showing that the pHRR72 protein contains the IgG constant region, and

that the 68 kD band seen under reducing conditions shifts to a high molecular weight dimeric form under non-reducing conditions. Thus since only dimeric IgG binds protein A, and since the mobility under non-reducing conditions is at least twice that of the monomer, we conclude that the tICAM(185)/IgG immunoadhesin is a dimer. Correct folding of the ICAM-1 region is indicated by the specific immunoprecipitation with c78.4A as in Example 6, and by the quantitative detection of the fusion protein using two ICAM-1-specific antibodies in a radioimmune assay (RIA) as in Example 4.

pHRR72 was co-transfected with pSV2-DHFR into CHO cells by the calcium phosphate method of Example 4 and DHFR+ cells were selected in nucleoside-free medium. Individual colonies were picked, expanded and tested by RIA for expression. The three highest-expressing colonies were selected for further study and were recloned by limiting dilution. Analysis of labelled cell supernatants by protein A binding and gel electrophoresis confirmed the expression of tICAM(185)/IgG dimers.

In a similar manner, domains I - V of ICAM-1 (residues 1-453) were linked to a segment of human immunoglobulin heavy chain cDNA. A fragment coding for residues 1-453 of ICAM-1 and a fragment coding for IgG heavy chain beginning at residue 216 in the hinge region and ending at the C-terminus of the molecule were each separately amplified. The PCR primer used at the 3' end of the ICAM-1 fragment contained an additional 24 bases complementary to the first 24 bases of the IgG fragment: CGG TGG GCA TGT GTG AGT TTT GTC CTC ATA CCG GGG GGA GAG CAC ATT. The 5' ICAM-1 primer, 5' IgG primer, and 3' primer from the end of the IgG coding sequence were the same as for the tICAM(185)IgG fusion above. After PCR amplification, a band of approximately 2000 bp consistent with a tICAM(453)/IgG fusion was produced.

Clones containing the desired insert were identified by restriction analysis and the clone designated pHRR 95-9 was selected for sequence analysis. The cDNA sequence is as follows:

	1	CAGACATCTG	TGTCCCCCTC	AAAAGTCATC	CTGCCCCGGG	GAGGCTCCGT
	51	GCTGGTGACA	TGCAGCACCT	CCTGTGACCA	GCCCAAGTTG	TTGGGCATAG
	101	AGACCCCGTT	GCCTAAAAAG	GAGTTGCTCC	TGCCTGGGAA	CAACCGGAAG
	151	GTGTATGAAC	TGAGCAATGT	GCAAGAAGAT	AGCCAACCAA	TGTGCTATTC
5	201	AAACTGCCCT	GATGGGCAGT	CAACAGCTAA	AACCTTCCTC	ACCGTGTACT
	251	GGACTCCAGA	ACGGGTGGAA	CTGGCACCCC	TCCCCTCTTG	GCAGCCAGTG
	301	GGCAAGAACC	TTACCCTACG	CTGCCAGGTG	GAGGGTGGGG	CACCCCGGGC
	351	CAACCTCACC	GTGGTGCTGC	TCCGTGGGGA	GAAGGAGCTG	AAACGGGAGC
	401	CAGCTGTGGG	GGAGCCCGCT	GAGGTCACGA	CCACGGTGCT	GGTGAGGAGA
10	451	GATCACCATG	GAGCCAATTT	CTCGTGCCGC	ACTGAACTGG	ACCTGCGGCC
	501	CCAAGGGCTG	GAGCTGTTTG	AGAACACCTC	GGCCCCCTAC	CAGCTCCAGA
	551	CCTTTGTCCT	GCCAGCGACT	CCCCCACAAC	TTGTCAGCCC	CCGGGTCCTA
	601	GAGGTGGACA	CGCAGGGGAC	CGTGGTCTGT	TCCCTGGACG	GGCTGTTCCC
	651	AGTCTCGGAG	GCCCAGGTCC	ACCTGGCACT	GGGGGACCAG	AGGTTGAACC
15	701	CCACAGTCAC	CTATGGCAAC	GACTCCTTCT	CGGCCAAGGC	CTCAGTCAGT
	751	GTGACCGCAG	AGGACGAGGG	CACCCAGCGG	CTGACGTGTG	CAGTAATACT
	801	GGGGAACCAG	AGCCAGGAGA	CACTGCAGAC	AGTGACCATC	TACAGCTTTC
	851	CGGCGCCCAA	CGTGATTCTG	ACGAAGCCAG	AGGTCTCAGA	AGGGACCGAG
	901	GTGACAGTGA	AGTGTGAGGC	CCACCCTAGA	GCCAAGGTGA	CGCTGAATGG
20	951	GGTTCCAGCC	CAGCCACTGG	GCCCGAGGGC	CCAGCTCCTG	CTGAAGGCCA
	1001	CCCCAGAGGA	CAACGGGCGC	AGCTTCTCCT	GCTCTGCAAC	CCTGGAGGTG
	1051	GCCGGCCAGC	TTATACACAA	GAACCAGACC	CGGGAGCTTC	GTGTCCTGTA
	1101	TGGCCCCCGA	CTGGACGAGA	GGGATTGTCC	GGGAAACTGG	ACGTGGCCAG
	1151	AAAATTCCCA	GCAGACTCCA	ATGTGCCAGG	CTTGGGGGAA	CCCATTGCCC
25	1201	GAGCTCAAGT	GTCTAAAGGA	TGGCACTTTC	CCACTGCCCA	TCGGGGAATC
	1251	AGTGACTGTC	ACTCGAGATC	TTGAGGGCAC	CTACCTCTGT	CGGGCCAGGA
	1301	GCACTCAAGG	GGAGGTCACC	CGCAAGGTGA	CCGTGAATGT	GCTCTCCCCC
	1351	CGGTATGAGg	acaaaactca	cacatgccca	ccgtgcccag	cacctgaact
	1401	cctgggggga	ccgtcagtct	tcctcttccc	cccaaaaccc	aaggacaccc
30	1451	tcatgatctc	ccggacccct	gaggtcacat	gcgtggtggt	ggacgtgagc
	1501	cacgaagacc	ctgaggtcaa	gttcaactgg	tacgtggacg	gcgtggaggt
	1551	gcataatgcc	aagacaaagc	cgcgggagga	gcagtacaac	agcacgtacc
	1601	gggtggtcag	cgtcctcacc	gtcctgcacc	aggactggct	gaatggcaag
	1651	gagtacaagt	gcaaggtctc	caacaaagcc	ctcccagccc	ccatcgagaa
35	1701	aaccatctcc	aaagccaaag	ggcagccccg	agaaccacag	gtgtacaccc
	1751	tgcccccatc	ccgggatgag	ctgaccaaga	accaggtcag	cctgacctgc
	1801	ctggtcaaag	gcttctatcc	cagcgacatc	gccgtggagt	gggagagcaa
	1851	tgggcagccg	gagaacaact	acaagaccac	gcctcccgtg	ctggactccg
	1901	acggctcctt	cttcctctac	agcaagctca	ccgtggacaa	gagcaggtgg
40	1951	cagcagggga	acgtcttctc	atgctccgtg	atgcatgagg	ctctgcacaa
	2001	ccactacacg	cagaagagcc	tctccctgtc	tccgggtaaa	tga

The corresponding amino acid sequence of the mature fusion polypeptide is as follows:

```
1    QTSVSPSKVI LPRGGSVLVT CSTSCDQPKL LGIETPLPKK ELLLPGNNRK
51   VYELSNVQED SQPMCYSNCP DGQSTAKTFL TVYWTPERVE LAPLPSWQPV
101  GKNLTLRCQV EGGAPRANLT VVLLRGEKEL KREPAVGEPA EVTTTVLVRR
151  DHHGANFSCR TELDLRPQGL ELFENTSAPY QLQTFVLPAT PPQLVSPRVL
201  EVDTQGTVVC SLDGLFPVSE AQVHLALGDQ RLNPTVTYGN DSFSAKASVS
251  VTAEDEGTQR LTCAVILGNQ SQETLQTVTI YSFPAPNVIL TKPEVSEGTE
301  VTVKCEAHPR AKVTLNGVPA QPLGPRAQLL LKATPEDNGR SFSCSATLEV
351  AGQLIHKNQT RELRVLYGPR LDERDCPGNW TWPENSQQTP MCQAWGNPLP
401  ELKCLKDGTF PLPIGESVTV TRDLEGTYLC RARSTQGEVT RKVTVNVLSP
451  RYEDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS
501  HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK
551  EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNQVSLTC
601  LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
651  QQGNVFSCSV MHEALHNHYT QKSLSLSPGK *
```

The plasmids were transfected into COS cells which were labelled with [^{35}S]cysteine overnight at 48 hours post-transfection as in Example 6. The fusion polypeptide is expressed as a soluble secreted disulfide-linked dimer which binds protein A. The supernatants were immunoprecipitated with anti-ICAM-1 monoclonal antibody c78.4A and analyzed by SDS gel electrophoresis as in Example 6. Under reducing conditions a band with an apparent molecular weight of 100 kD was specifically immunoprecipitated, corresponding to the ICAM-1/IgG fusion, while under non-reducing conditions it migrates as a 200 kD dimer.

EXAMPLE 13

Rhinovirus Binding and Neutralization by tICAM/IgG Immunoadhesins

The tICAM(185)/IgG immunoadhesin of Example 12 consists of residues 1-185 of ICAM-1 fused to residue 216 in the hinge region of an IgG1 heavy chain. The molecule is a disulfide-linked dimer containing two rhinovirus binding sites. A CHO cell line CHO72.2 secreting the immunoadhesin was grown overnight in serum-free media containing [^{35}S]cysteine and the fusion protein was purified on protein A beads. The labelled protein was tested for rhinovirus binding in the pelleting assay as described in Example 7(A). The samples consisted of tICAM(185)/IgG (no virus),

tICAM(185)/IgG + HRV3, tICAM(185)/IgG + HRV3 + c78.4A, and tICAM(185)/IgG + HRV3 + irrelevant antibody. Pelleting of labelled protein indicative of virus binding was seen with virus and virus + irrelevant antibody by analysis on SDS gels. No pelleting was seen in the absence of virus and significantly reduced pelleting was seen in the sample containing c78.4A. This result indicates that the tICAM(185)/IgG binds rhinovirus with a significantly higher affinity than the soluble monomers tICAM(185) and tICAM(453), which do not show levels of binding readily detectable under these conditions. See Example 7(A). While approximately 10% of tmICAM-1 pellets under these conditions, only 1% of tICAM(453) pellets, presumably because tmICAM-1 is in a dimeric state. The result with tICAM(185)/IgG is similar to that seen in this assay with tmICAM-1, suggesting that the two forms of ICAM may have similar affinities for the virus, and providing further evidence that tmICAM-1 is a dimer.

Cell supernatant from CHO72.2 cells containing unpurified tICAM(185)/IgG was tested for rhinovirus neutralization in a virus infectivity assay according to the method of Example 10(B). Serial dilutions of HRV3 were made in media containing 50% IgG supernatant or control supernatant from untransfected CHO cells. The virus dilutions were mixed with HeLa cells and plated in wells of a 96-well microtiter plate (10 wells per dilution). Virus titers were determined by scoring the number of infected wells at each dilution after 6 days. In addition a quantitative assessment of cytopathic effect at high virus input was made 2 days after infection. In experiment A the concentration of tICAM(185)/IgG estimated by RIA was 150 ng/ml and in experiment (B) the concentration was 325 ng/ml.

TABLE 6

	Experiment A	Experiment B
HRV3	1×10^7 PFU/ml	4×10^6 PFU/ml
HRV3 + tICAM(185)/IgG	6×10^5 PFU/ml	5×10^5 PFU/ml

Both experiments resulted in a ten-fold reduction in viral titer at a concentration of approximately 1 nM in experiment A and 2 nM in experiment B. For comparison, monomeric tICAM(453) in the same assay results in a 50% reduction in titer at 0.38 μM or 30 μg/ml. Thus the increase in activity resulting from dimerization of the rhinovirus binding site is at least 200-fold and probably greater.

Cell supernatant from CHO72.2 at a concentration of 650 ng/ml (4 nM) was also tested in a competitive binding assay measuring the binding of [^{35}S]HRV3 to ICAM-1-coated plastic microtiter wells. Specific binding is determined by comparing counts bound with or without pre-incubation of the ICAM-1 in the well with Mab c78.4A.

TABLE 7

	cpm bound*	% binding
HRV3	4945 +/- 58	100
HRV3 + CHO supernatant	5358 +/- 51	108
HRV3 + CHO72.2 supernatant	3187 +/- 206	64

*Mean values determined from triplicate wells. Standard errors were less than 10% of the mean.

The level of binding in the presence of tICAM(185)/IgG was 65% of the normal control binding and 54% of control binding in the presence of CHO cell supernatant, indicating close to a 50% inhibition of binding. For comparison, soluble monomeric tICAM(453) inhibits HRV3 binding by 50% in the same assay at 240 μg/ml or 3.1 μM. On a molar basis the ICAM-1 IgG immunoadhesin was thus almost a 1000-fold better competitor than the monomer. The above experiments were done with supernatants. Subsequent attempts to reproduce these results with highly purified tICAM(185)/IgG were unsuccessful.

The tICAM(453)/IgG immunoadhesin of Example 12 consists of residues 1-453 of ICAM-1 fused to residue 216 in the hinge region of an IgG1 heavy chain. The molecule is a disulfide-linked dimer containing two rhinovirus binding sites. The fusion polypeptide was expressed in HeLa cells using the vaccinia/T7 system and purified from the supernatant by affinity chromatography using an anti-ICAM-1

monoclonal antibody. The activity of the protein was examined in a competitive binding assay which measures the binding of [^{35}S]-labelled HRV to plates coated with purified tmICAM-1. For comparison, soluble monomeric tICAM-453 was included in a parallel assay as a positive control. The binding values are documented in Table 8 below:

TABLE 8

	IC_{50}*
tICAM(453)	44 nM
t(453)/IgG	
Experiment 1	11 nM
Experiment 2	10 nM

*IC_{50} is the concentration required to inhibit binding by 50%

These values are per mol of tICAM(453) determined by RIA. Since each fusion polypeptide contains two tICAM(453) polypeptides, the values for the fusion polypeptide expressed per mol of dimer are 5.5 nM and 5 nM for Experiments 1 and 2, respectively. Therefore on a molar basis the activity of the fusion polypeptide in the competitive binding assay is ten-fold greater than the tICAM(453) monomer. In subsequent experiments the relative activity was 2- to 4-fold greater.

EXAMPLE 14

In Vitro Dimerization of ICAM-1

Several lines of evidence indicate that tmICAM-1 exists as a noncovalent dimer at the cell surface: (i) the stoichiometry of HRV/ICAM-1 binding sites at the cell surface is approximately 2; (ii) tICAM(453), despite being properly folded, has a approximately 100-fold lower affinity for HRV than purified tmICAM-1; and (iii) tICAM(453) and tmICAM-1 absorbed to nitrocellulose filters at a high density bind rhinovirus at equivalent levels. See Example 7. In addition, Staunton et al. (Cell

61:243-254 (1990)) have reported that some mutants of ICAM-1 form covalent dimers at the cell surface, indicating that this protein has the capability to self-associate in vivo. Attempts to directly demonstrate the existence of dimers by chemical crosslinking with water-soluble carbodiimide/NHS, which is a heterobifunctional crosslinker which forms a covalent bond between a primary amine and a carboxyl group, did result in crosslinking of tICAM(453) into a 180 kD species, whose size is consistent with a dimer (Figure 4A). This crosslinking is directly dependent upon the concentration of tICAM(453), with 50% crosslinking at 7 μM protein (Figure 4B). This concentration is consistent with the relatively high concentration of tmICAM-1 at the surface of a HeLa cell, which is approximately 2.5 μM or 135 μg/ml. The self-association detected by this crosslinking is specific, since it is not affected by high concentrations of third-party proteins (Figure 4C). tICAM(185) appears to be poorly crosslinked under the same conditions, indicating that domains 3-5 are involved in self-association. Because of the extensive modification of the protein by this crosslinking procedure, the protein had no virus-binding activity. However, this data shows that soluble ICAM can self-associate in solution, and that this self-association is concentration-dependent and -specific.

EXAMPLE 15

A tICAM(1-451)/LFA-3(210-237) Chimera

In order to examine the role of the transmembrane and cytoplasmic domains of tmICAM-1 in high-affinity rhinovirus binding, we constructed a chimeric ICAM-1 which is anchored on the cell surface by a phospholipid tail and lacks these domains (see Fig. 5). This experiment was designed to test whether the cytoplasmic and transmembrane domains are necessary for the formation of dimeric ICAM-1 on the cell surface, which results in the high affinity binding of rhinovirus. In order to modify the ICAM-1 cDNA to express a phospholipid-anchored form, we first used site-directed mutagenesis to create a unique SacII site at residues 450/451 close to the end of the extracellular region. This allowed the isolation of a cDNA fragment coding for residues 1-451 of ICAM-1, by digestion of the modified plasmid with HindIII and SacII. We used PCR to generate a fragment coding for the C-terminal

28 amino acids of the phospholipid-anchored form of LFA-3 (Seed, B., Nature (1987) 329:840-842). By including a SacII site in the 5' primer this fragment was ligated to the ICAM-1 extracellular domain and cloned into the expression vector CDM8, resulting in the plasmid pHRR 70-19. This plasmid contains a cDNA coding for residues 1-451 of ICAM-1 fused to residues 210-237 of LFA-1, which should result in the expression of a phosphoplipid-anchored molecule containing the ICAM-1 extracellular region. See Fig. 5.

Transfection of COS cells with pHRR 70-19 according to the method of Example 4 and FACS analysis with anti-ICAM-1 antibodies confirmed the cell surface expression of the fusion protein. The binding of [^{35}S]-labelled cells to COS cells transfected with the fusion protein was determined.

TABLE 9

ICAM-1	cpm bound	% virus input	% control
tmICAM-1	2130 +/- 278	9.4	100
tICAM(1-185)/ LFA-3(210-237) chimera	2382 +/- 293	11.2	119

This result shows that there is no significant difference between the ability of tmICAM-1 and the tICAM(1-451)/LFA-3(210-237) chimera to bind HRV. It can therefore be concluded that the transmembrane and cytoplasmic domains are not required for HRV binding, and that dimerization must depend on interactions between extracellular regions of the molecule.

Additional evidence that a form of ICAM-1 lacking the cytoplasmic and transmembrane domains functions efficiently as a receptor for rhinoviruses was obtained by transfection of the tICAM(1-451)/LFA-3(210-237) chimeric gene into HeLa 229 cells. We have determined that these cells do not express ICAM-1 on the surface and are resistant to HRV infection. Transfection of either tmICAM-1 or the

tICAM(1-451)/LFA-3(210-237) chimera results in cells which are readily infectable with rhinovirus and produce virus at levels comparable to normal HeLa cells.

EXAMPLE 16

Irreversible Inactivation of HRV by ICAM

We have demonstrated that tICAM(453) can, in addition to blocking the binding of HRV to cells, irreversibly inactivate HRV. Incubation of HRV with tICAM(453) at 34 C results in conversion of a fraction of the virus from the native 148S form to a 42S form (Figure 6). The 42S form is non-infectious, lacks the viral subunit VP4, and lacks the RNA genome (empty capsid). This can be shown by SDS-PAGE analysis of [^{35}S]methionine-labelled viral particles and by quantitation of viral RNA content by hybridization with a [^{32}P]oligonucleotide probe for rhinovirus (5'-GCATTCAGGGGCCGGAG-3'). Thus, tICAM(453) can uncoat rhinovirus, an event that normally occurs intracellularly during the course of infection. The uncoating is a slow process, occurring with a t1/2 of 6 hours at 34 C, in contrast with the inhibition of binding, which occurs with a t1/2 of <30 minutes. The uncoating is highly temperature-dependent, occurring 10 times faster at 37 C than at 34 C, the optimal temperature of rhinovirus growth. Enhancement of this uncoating activity by soluble forms of ICAM-1 including multimeric configurations of ICAM-1 will lead to improvement of antiviral activity by making neutralization irreversible.

Example 17

Cysteine Muteins

To identify the correct site to place cysteine residues for multimerization of ICAM-1, the region of the protein surface involved in self-association must be identified. Domains IV and V have been chosen because they are distal to the viral binding sites (domain I) and because domains II-V are implicated in self-association (see Example 14). Since the structure of ICAM-1 is not certain, we have attempted to align the sequence of domains IV and V at the C-terminus of the extracellular domain of ICAM-1 onto the immunoglobulin fold, as ICAM-1 has homology to

members of the immunoglobulin supergene family. This alignment is shown diagrammatically in Fig. 7. Then, to identify probable sites involved in self-association, we have examined the three-dimensional structures of several members of the immunoglobulin supergene family, IgG and MHC1/beta-2 microglobulin. Immunoglobulin domains have two broad faces of beta sheet structure, here designated the "B" face and the "F" face. Inspection of the above structures revealed that different immunoglobulin-like domains interacted via one or the other of these faces of the domain. IgG variable regions associated via their F face, while IgG constant regions (CH1, CH2, and CH3) and MHC1/beta-2 microglobulin all interact via their B faces.

ICAM-1 domains have highest homology to constant region-like domains. Thus, the most likely sites of interaction are on the B face of the domains; the most likely sites on the B face to place cysteine residues are close to the center of the B face (adjacent to the cysteine on the B strand that forms the intrachain disulfide bond), where IgG CH3 domains self-associate, or on the N-terminal end of the B face, where IgG CH2 domains and MHC1/beta-2 microglobulin self-associate.

A number of mutants were prepared to identify appropriate sites of interaction. These mutants were prepared by standard site-directed mutagenesis methodology to mutate selected residues to cysteine on tICAM(453) and tmICAM. These cDNAs in the vector CDM8 were then transfected into COS cells and dimer formation accessed by biosynthetic labelling of ICAM-1 with [^{35}S]cysteine followed by immunoprecipitation and non-reducing SDS-PAGE analysis. As shown in Table 10, of 13 mutants tested, two have been found to form dimers at a small (about 5%) but significant level:

TABLE 10

Position of Cysteine	Dimer Formation
(tmICAM-1)	
304	-
306	-
307	+
309	+
375	-
377	-
378	-
380	-
382	-
429	-
(tICAM(453))	
338	-
360	-
378	-

These two muteins, Cys-307 and Cys-309, are both located on the N-terminal end of the B face of domain IV. The relatively low level of dimerization may reflect the low concentration of ICAM-1 on the cell surface (low expression), or imperfect orientation of the cysteine residues relative to the site of interaction. These data indicate that this region of the domain is a likely site of interaction. Other residues adjacent to residues 307 and 309, e.g. His-308, Arg-310, Glu-294, Arg-326, Gln-328, are likely to increase the efficiency of the dimer formation. Mutations that lead to dimer formation of tmICAM-1 are then be placed on tICAM(453) for the secretion of soluble ICAM-1 dimers.

A tICAM(452) cysteine mutant was prepared by substituting a cysteine for an alanine at position 307 in the ICAM-1 amino acid sequence and inserting a stop codon

after amino acid residue 452. The mutein was constructed by site-directed mutagenesis using a full-length ICAM-1 cDNA and has the following DNA sequence:

```
1     CAGACATCTG  TGTCCCCCTC  AAAAGTCATC  CTGCCCCGGG  GAGGCTCCGT
51    GCTGGTGACA  TGCAGCACCT  CCTGTGACCA  GCCCAAGTTG  TTGGGCATAG
101   AGACCCCGTT  GCCTAAAAAG  GAGTTGCTCC  TGCCTGGGAA  CAACCGGAAG
151   GTGTATGAAC  TGAGCAATGT  GCAAGAAGAT  AGCCAACCAA  TGTGCTATTC
201   AAACTGCCCT  GATGGGCAGT  CAACAGCTAA  AACCTTCCTC  ACCGTGTACT
251   GGACTCCAGA  ACGGGTGGAA  CTGGCACCCC  TCCCCTCTTG  GCAGCCAGTG
301   GGCAAGAACC  TTACCCTACG  CTGCCAGGTG  GAGGGTGGGG  CACCCCGGGC
351   CAACCTCACC  GTGGTGCTGC  TCCGTGGGGA  GAAGGAGCTG  AAACGGGAGC
401   CAGCTGTGGG  GGAGCCCGCT  GAGGTCACGA  CCACGGTGCT  GGTGAGGAGA
451   GATCACCATG  GAGCCAATTT  CTCGTGCCGC  ACTGAACTGG  ACCTGCGGCC
501   CCAAGGGCTG  GAGCTGTTTG  AGAACACCTC  GGCCCCCTAC  CAGCTCCAGA
551   CCTTTGTCCT  GCCAGCGACT  CCCCCACAAC  TTGTCAGCCC  CCGGGTCCTA
601   GAGGTGGACA  CGCAGGGGAC  CGTGGTCTGT  TCCCTGGACG  GGCTGTTCCC
651   AGTCTCGGAG  GCCCAGGTCC  ACCTGGCACT  GGGGGACCAG  AGGTTGAACC
701   CCACAGTCAC  CTATGGCAAC  GACTCCTTCT  CGGCCAAGGC  CTCAGTCAGT
751   GTGACCGCAG  AGGACGAGGG  CACCCAGCGG  CTGACGTGTG  CAGTAATACT
801   GGGGAACCAG  AGCCAGGAGA  CACTGCAGAC  AGTGACCATC  TACAGCTTTC
851   CGGCGCCCAA  CGTGATTCTG  ACGAAGCCAG  AGGTCTCAGA  AGGGACCGAG
901   GTGACAGTGA  AGTGTGAGtg  CCACccgcgg  GCCAAGGTGA  CGCTGAATGG
951   GGTTCCAGCC  CAGCCACTGG  GCCCGAGGGC  CCAGCTCCTG  CTGAAGGCCA
1001  CCCCAGAGGA  CAACGGGCGC  AGCTTCTCCT  GCTCTGCAAC  CCTGGAGGTG
1051  GCCGGCCAGC  TTATACACAA  GAACCAGACC  CGGGAGCTTC  GTGTCCTGTA
1101  TGGCCCCCGA  CTGGACGAGA  GGGATTGTCC  GGGAAACTGG  ACGTGGCCAG
1151  AAAATTCCCA  GCAGACTCCA  ATGTGCCAGG  CTTGGGGGAA  CCCATTGCCC
1201  GAGCTCAAGT  GTCTAAAGGA  TGGCACTTTC  CCACTGCCCA  TCGGGGAATC
1251  AGTGACTGTC  ACTCGAGATC  TTGAGGGCAC  CTACCTCTGT  CGGGCCAGGA
1301  GCACTCAAGG  GGAGGTCACC  CGCAAGGTGA  CCGTGAATGT  GCTCTCCCCC
1351  CGGTATTAG
```

The foregoing examples describe the creation of soluble, multimeric forms of tICAM that substantially increase tICAM binding and neutralizing activity.

While the present invention has been described in terms of specific methods and compositions, it is understood that variations and modifications will occur to those skilled in the art upon consideration of the present invention.

For example, it is anticipated that smaller protein fragments and peptides derived from ICAM-1 that still contain the virus-binding site would also be effective in a multimeric configuration. It is also anticipated that multimeric ICAM may be effective inhibitors of the ICAM-1/LFA-1 interaction, as the affinity between these

effective inhibitors of the ICAM-1/LFA-1 interaction, as the affinity between these two molecules is quite low and the cell-cell binding mediated by these two molecules is highly cooperative.

Although the preferred form and configuration is a non-transmembrane (truncated) ICAM in dimeric configuration, it is not intended to preclude other forms and configurations effective in binding virus and effective in neutralizing viral activity from being included in the scope of the present invention.

Further, it is anticipated that the general method of the invention of preparing soluble protein forms from insoluble, normally membrane bound receptor proteins can be used to prepare soluble multimeric forms of other receptor proteins useful for binding to and decreasing infectivity of viruses other than those that bind to the "major group" receptor. Such other viruses include polio, Herpes simplex, and Epstein-Barr virus.

Numerous modifications and variations in the invention as described in the above illustrative examples are expected to occur to those skilled in the art and consequently only such limitations as appear in the appended claims should be placed thereon.

Accordingly it is intended in the appended claims to cover all such equivalent variations which come within the scope of the invention as claimed.

WHAT IS CLAIMED IS:

1. Multimeric ICAM.

2. The multimeric ICAM of claim 1 wherein said ICAM is non-transmembrane ICAM.

3. The multimeric ICAM of claim 2 wherein said non-transmembrane ICAM is substantially without the carboxyl intracellular domain and without the hydrophobic membrane domain.

4. The multimeric ICAM according to claim 2 wherein said non-transmembrane ICAM is a member selected from the group consisting of tICAM(453), tICAM(185), tICAM(88), tICAM(283), and tICAMs comprising one or more sequences selected from tICAM(89-185), tICAM186-283, tICAM(284-385), tICAM(386-453), tICAM(75-77), tICAM(70-72), tICAM(64-66), tICAM(40-43), tICAM(36-38), tICAM(30-33), and tICAM(26-29).

5. The multimeric ICAM of claim 1 wherein said ICAM is multimerized by adsorption to a support.

6. The multimeric ICAM of claim 5 wherein said support is an inert polymer and is a member selected from the group consisting of nitrocellulose, PVDF, DEAE, lipid polymer, and amino dextran.

7. The multimeric ICAM of claim 1 wherein said multimeric ICAM is multimerized by coupling to a member.

8. The multimeric ICAM of claim 7 wherein said ICAM is modified with at least one reactive amino acid to provide at least one site to facilitate coupling.

9. The multimeric ICAM of claim 8 wherein said reactive amino acid is a member selected from the group consisting of lysine and cysteine.

10. The multimeric ICAM of claim 7 wherein said member is a member selected from the group consisting of an antibody and a protein carrier.

11. The multimeric ICAM of claim 10 wherein said antibody is anti-ICAM antibody CL 203.

12. The multimeric ICAM of claim 10 wherein said protein carrier is a member selected from the group consisting of albumin and proteoglycans.

13. The multimeric ICAM of claim 1 wherein said ICAM is modified at either terminus to comprise a lipid capable of promoting formation of oligomer micelles.

14. The multimeric ICAM of claim 1 comprising two or more ICAMs, which may be the same or different, linked to each other.

15. The multimeric ICAM of claim 14 wherein said ICAMs are directly linked to each other without a linker.

16. The multimeric ICAM of claim 15 wherein said ICAMs are linked to each other via at least one disulfide bridge.

17. The multimeric ICAM of claim 16 wherein said ICAMs are crosslinked via a cysteine disulfide bridge at position 307 on each ICAM.

18. The multimeric ICAM of claim 16 wherein said ICAMs are crosslinked via a cysteine disulfide bridge at position 309 on each ICAM.

19. The multimeric ICAM of claim 14 wherein said ICAMs are indirectly linked via a cross-linking agent.

20. The multimeric ICAM of claim 19 wherein said cross-linking agent is selected from the group consisting of heterobifunctional and homobifunctional cross-linking reagents.

21. The multimeric ICAM of claim 20 wherein said cross-linking reagent is a member selected from the group consisting of bifunctional N-hydroxysuccinimide esters, imidoesters and bis-maleimido-hexanes.

22. The multimeric ICAM of claim 1 wherein said ICAM is a member selected from the group consisting of fully glycosylated ICAM, partially glycosylated ICAM, or non-glycosylated ICAM.

23. In a method for enhancing the binding of ICAM to a ligand, the improvement comprising the steps of:

presenting said ICAM in a multimeric configuration.

24. The method according to claim 23 wherein said ICAM is tICAM.

25. The method according to claim 24 wherein said ICAM is a member selected from the group consisting of tICAM(453), tICAM(185), tICAM(88), tICAM(283), and tICAMs comprising one or more sequences selected from tICAM(89-185), tICAM186-283, tICAM(284-385), tICAM(386-453), tICAM(75-77), tICAM(70-72), tICAM(64-66), tICAM(40-43), tICAM(36-38), tICAM(30-33), and tICAM(26-29).

26. The method according to claim 23 wherein said ICAM is modified with at least one reactive amino acid to provide at least one site to facilitate coupling.

27. The method according to claim 26 wherein said reactive amino acid is selected from the group consisting of lysine and cysteine.

28. The method according to claim 23 wherein said ICAM is modified at either terminus to comprise a lipid capable of promoting formation of oligomer micelles.

29. The method according to claim 23 wherein said multimeric configuration comprises a first ICAM cross-linked to a second ICAM.

30. The method according to claim 29 wherein said first and second ICAM are each muteinized to contain a cysteine residue at position 307, and said first and second ICAM are cross-linked via a disulfide bridge between said cysteines at position 307.

31. The method according to claim 29 wherein said first and second ICAM are each muteinized to contain a cysteine residue at position 309, and said first and second ICAM are cross-linked via a disulfide bridge between said cysteines at position 309.

32. The method according to claim 23 wherein said multimeric configuration comprises ICAM adsorbed to a support.

33. The method according to claim 32 wherein said support comprises a member selected from the group consisting of high molecular weight and substantially inert polymers.

34. The method according to claim 33 wherein said polymer is an inert polymer and is a member selected from the group consisting of nitrocellulose, PVDF, DEAE, lipid polymers, and amino dextran.

35. The method according to claim 33 wherein said multimeric ICAM is multimerized by coupling to a member.

36. The method according to claim 35 wherein said member is a member selected from the group consisting of an antibody and a protein carrier.

37. The method according to claim 29 wherein said cross-linking reagent is a member selected from the group consisting of heterobifunctional and homobifunctional cross-linking reagents.

38. The method according to claim 37 wherein said protein carrier is a member selected from the group consisting of albumin and proteoglycans.

39. The method according to claim 36 wherein said antibody is anti-ICAM antibody CL 203.

40. The method according to claim 23, wherein said ligand is a member selected from the group consisting of human rhinovirus, major group receptor virus, lymphocyte-associated antigen-1 (LFA-l) and Plasmodium falciparum.

41. A pharmaceutical composition comprising a pharmaceutically acceptable solvent, diluent, adjuvant or a carrier, and, as the active ingredient, an effective amount of a polypeptide according to claim 1.

42. A method for inducing irreversible uncoating of human rhinovirus, said method comprising contacting said human rhinovirus with ICAM-1 or a tICAM fragment thereof.

43. A method of irreversibly inhibiting infectivity of a mammalian cell by a human rhinovirus, said method comprising contacting said human rhinovirus with ICAM-1 or a tICAM fragment thereof under conditions which allow the ICAM-1 or tICAM to bind to said rhinovirus; thereby stimulating irreversible uncoating of said rhinovirus.

Intercellular adhesion molecule-1(ICAM-1)

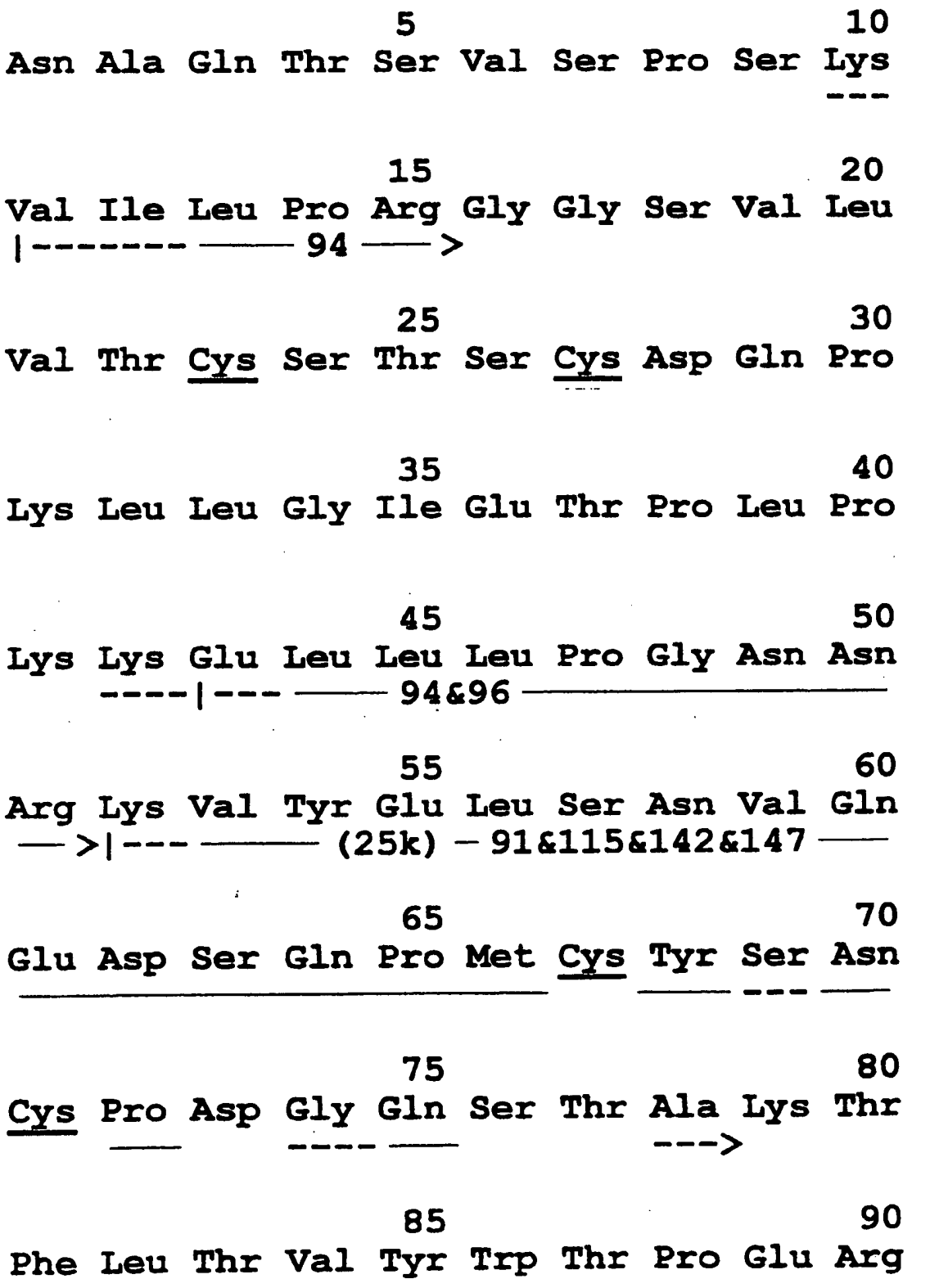


FIG. 1

```
                    95                  100
Val Glu Leu Ala Pro Leu Pro Ser Trp Gln

                   105                  110
Pro Val Gly Lys Asn Leu Thr Leu Arg Cys

                   115                  120
Gln Val Glu Gly Gly Ala Pro Arg Ala Asn

                   125                  130
Leu Thr Val Val Leu Leu Arg Gly Glu Lys
                                        ---

                   135                  140
Glu Leu Lys Arg Glu Pro Ala Val Gly Glu
|    ---------- (34k) - 103&114&121&135 ---

                   145                  150
Pro Ala Glu Val Thr Thr Thr Val Leu Val
---------------------------- (xx) ----------

                   155                  160
Arg Arg Asp His His Gly Ala Asn Phe Ser
------------    ------------    --->

                   165                  170
Cys Arg Thr Glu Leu Asp Leu Arg Pro Gln

                   175                  180
Gly Leu Glu Leu Phe Glu Asn Thr Ser Ala
```

FIG. 1 (CONT.)

```
                    185                 190
Pro Tyr Gln Leu Gln Thr Phe Val Leu Pro

                    195                 200
Ala Thr Pro Pro Gln Leu Val Ser Pro Arg
                                        ---

                    205                 210
Val Leu Glu Val Asp Thr Gln Gly Thr Val
| (x) ———————— (50k) – 110 ————————————

                    215                 220
Val Cys Ser Leu Asp Gly Leu Phe Pro Val
———  ————————————————————————————————

                    225                 230
Ser Glu Ala Gln Val His Leu Ala Leu Gly
----  ———————     ———     ----    --->

                    235                 240
Asp Gln Arg Leu Asn Pro Thr Val Thr Tyr

                    245                 250
Gly Asn Asp Ser Phe Ser Ala Lys Ala Ser

                    255                 260
Val Ser Val Thr Ala Glu Asp Glu Gly Thr

                    265                 270
Gln Arg Leu Thr Cys Ala Val Ile Leu Gly
```

FIG. 1 (CONT.)

```
                    275                 280
Asn Gln Ser Gln Glu Thr Leu Gln Thr Val

                    285                 290
Thr Ile Tyr Ser Phe Pro Ala Pro Asn Val

                    295                 300
Ile Leu Thr Lys Pro Glu Val Ser Glu Gly

                    305                 310
Thr Glu Val Thr Val Lys Cys Glu Ala His

                    315                 320
Pro Arg Ala Lys Val Thr Leu Asn Gly Val

                    325                 330
Pro Ala Gln Pro Leu Gly Pro Arg Ala Gln

                    335                 340
Leu Leu Leu Lys Ala Thr Pro Glu Asp Asn

                    345                 350
Gly Arg Ser Phe Ser Cys Ser Ala Thr Leu

                    355                 360
Glu Val Ala Gly Gln Leu Ile His Lys Asn
```

FIG. 1 (CONT.)

```
                    365                 370
Gln Thr Arg Glu Leu Arg Val Leu Tyr Gly

                    375                 380
Pro Arg Leu Asp Glu Arg Asp Cys Pro Gly

                    385                 390
Asn Trp Thr Trp Pro Glu Asn Ser Gln Gln

                    395                 400
Thr Pro Met Cys Gln Ala Trp Gly Asn Pro

                    405                 410
Leu Pro Glu Leu Lys Cys Leu Lys Asp Gly
                                   ----|------

                    415                 420
Thr Phe Pro Leu Pro Ile Gly Glu Ser Val
----  ------ 97&46 ------------------------

                    425                 430
Thr Val Thr Arg Asp Leu Glu Gly Thr Tyr
-------------------------------------  --

                    435                 440
Leu Cys Arg Ala Arg Ser Thr Gln Gly Glu
--  (xx) ---------  --->

                    445                 450
Val Thr Arg Glu Val Thr Val Asn Val Leu
```

FIG. 1 (CONT.)

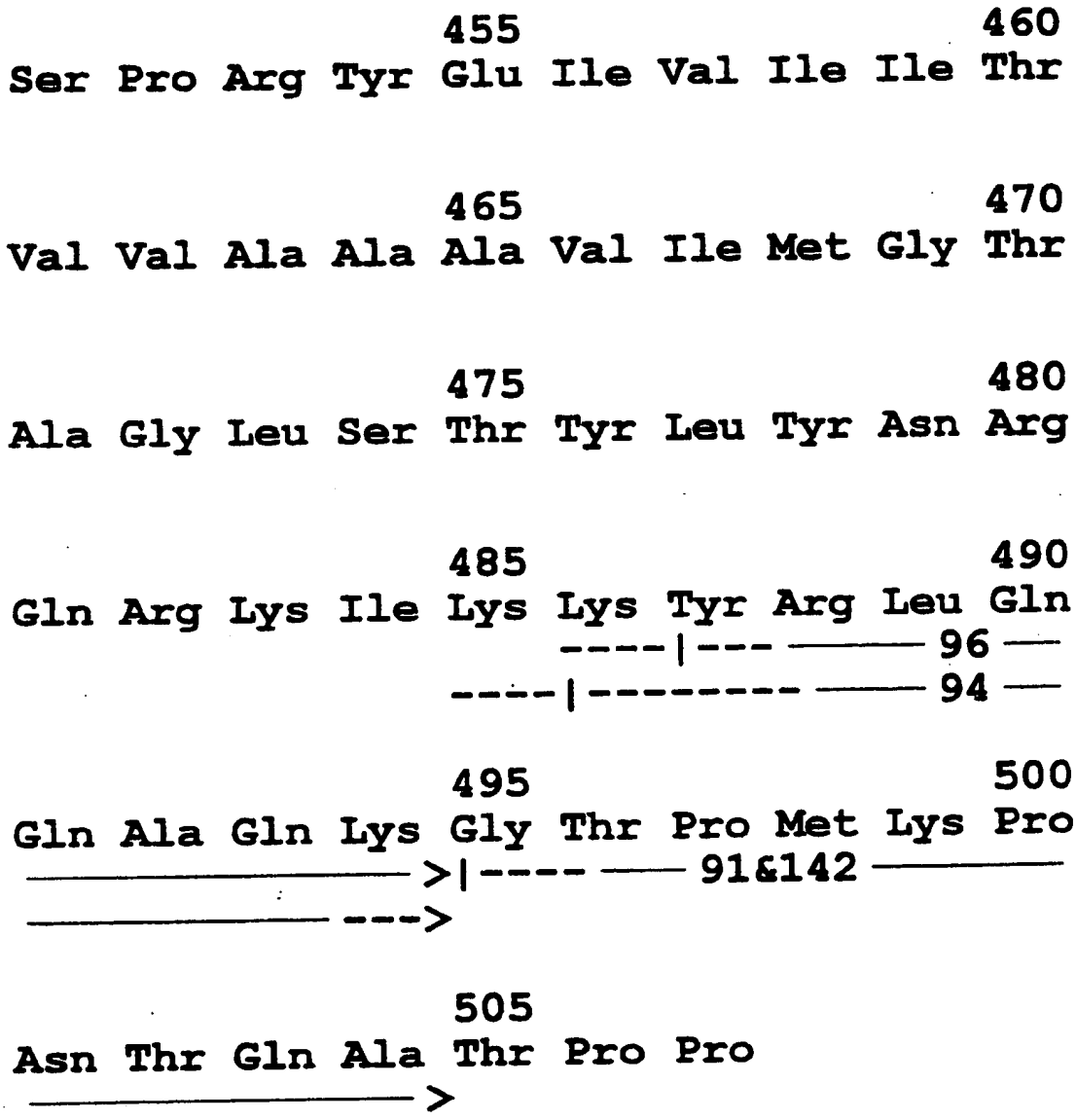

455
460
Ser Pro Arg Tyr Glu Ile Val Ile Ile Thr
465
470
Val Val Ala Ala Ala Val Ile Met Gly Thr
475
480
Ala Gly Leu Ser Thr Tyr Leu Tyr Asn Arg
485
490
Gln Arg Lys Ile Lys Lys Tyr Arg Leu Gln
96
94
495
500
Gln Ala Gln Lys Gly Thr Pro Met Lys Pro
91&142
505
Asn Thr Gln Ala Thr Pro Pro

FIG. 1 (CONT.)

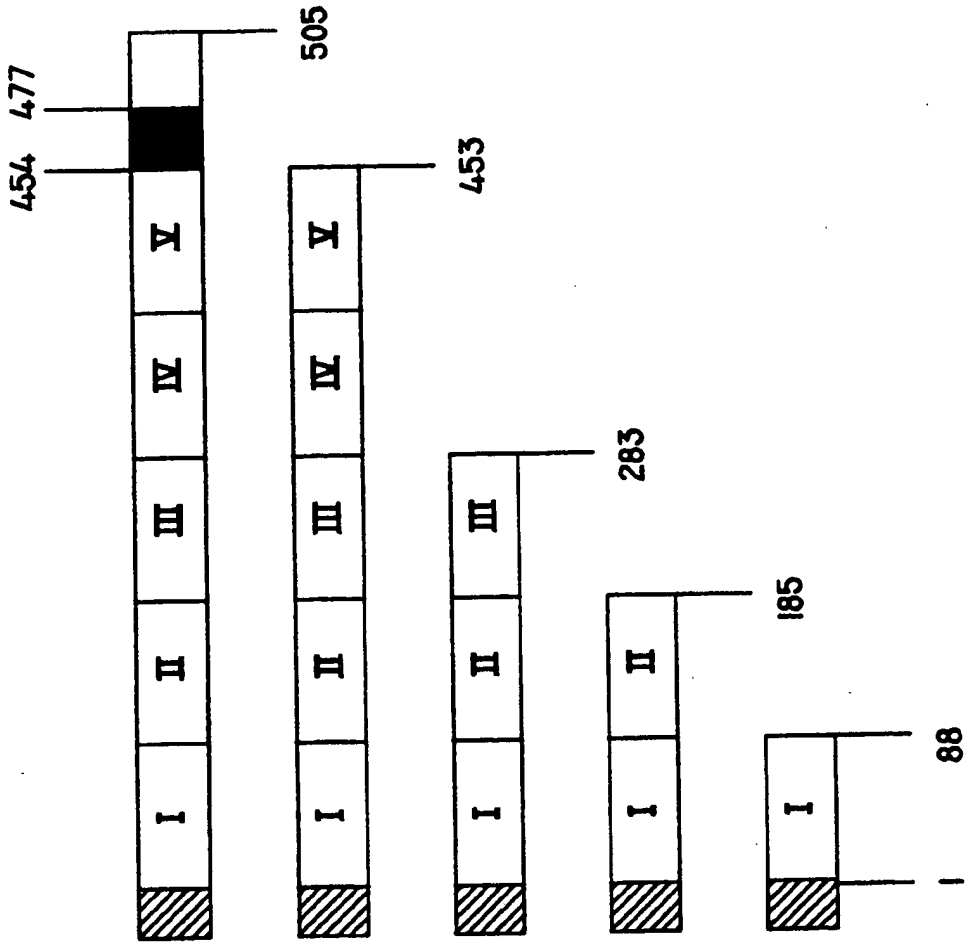


FIG 2A tmICAM-1

FIG. 2B tICAM(453)

FIG. 2C tICAM(283)

FIG. 2D tICAM(185)

FIG. 2E tICAM(88)

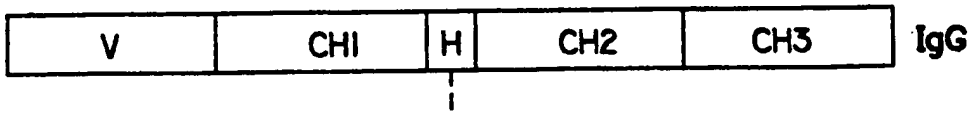


FIG. 3A

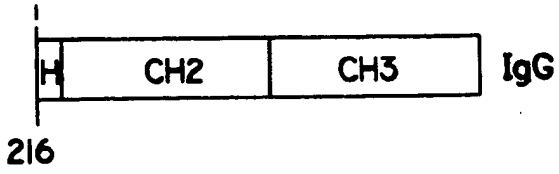


FIG. 3B

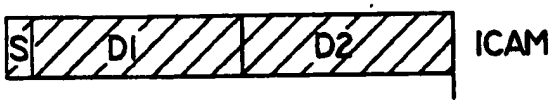


FIG. 3C

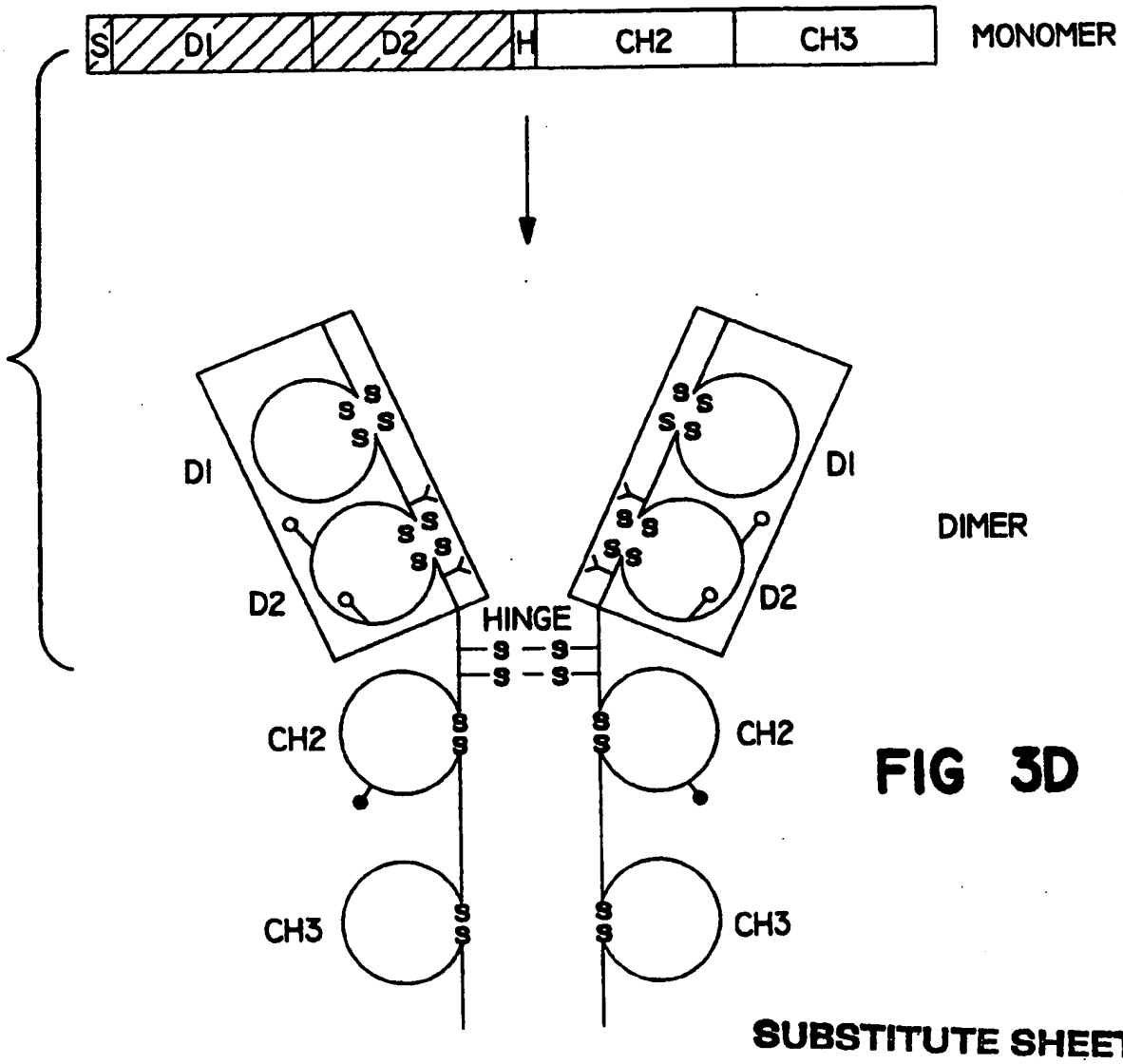


FIG 3D

FIG.4A

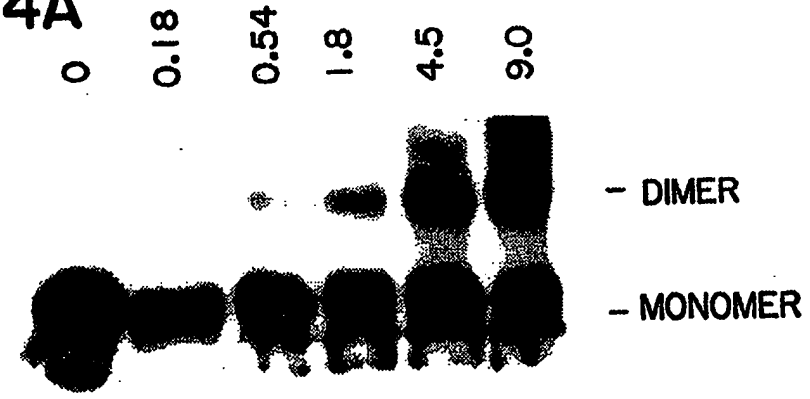


FIG.4B

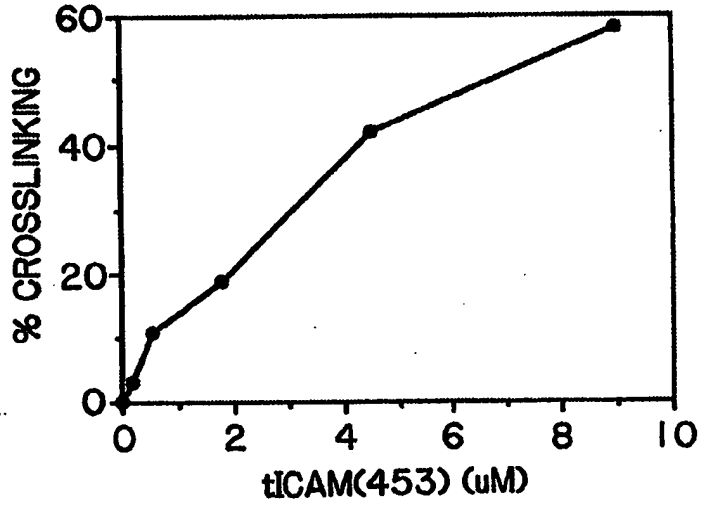
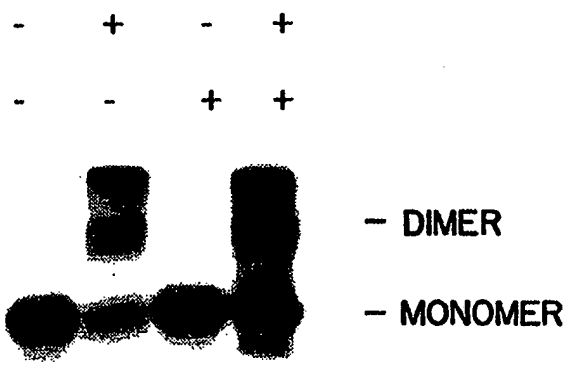


FIG.4C



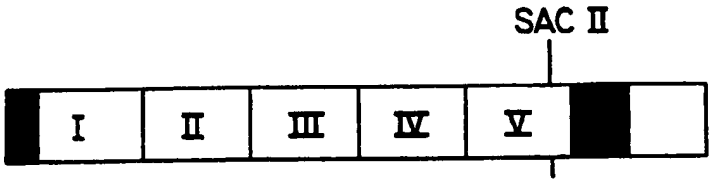


FIG. 5A

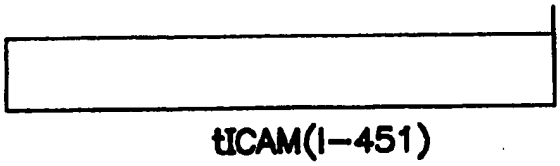
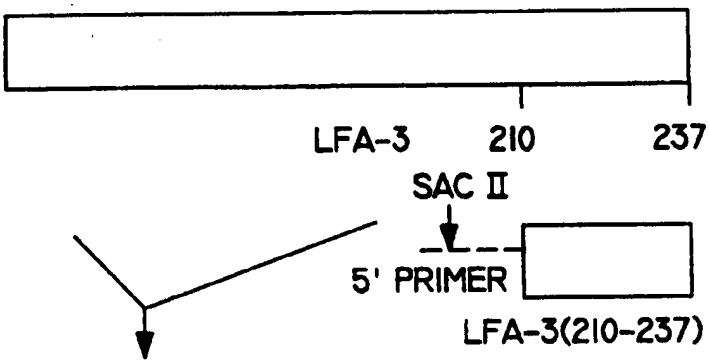


FIG. 5B

FIG. 5C



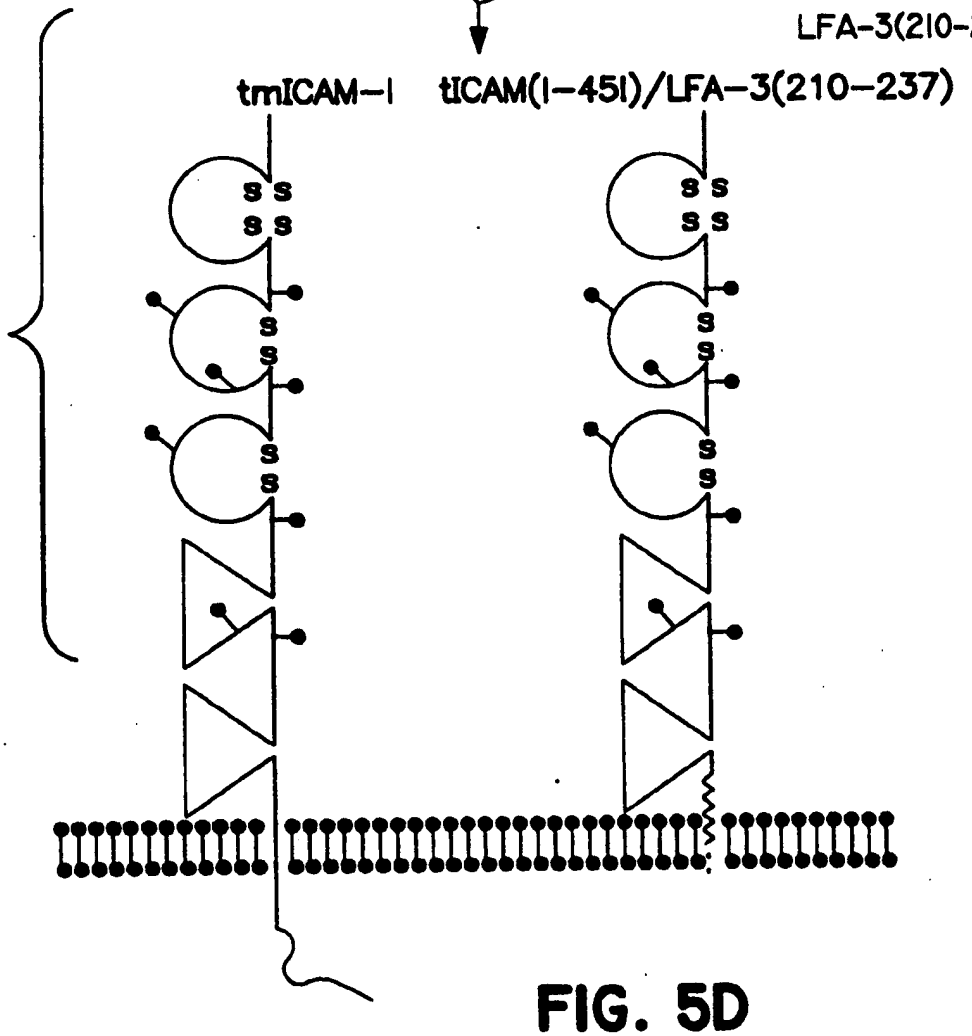


FIG. 5D

FIG.6A

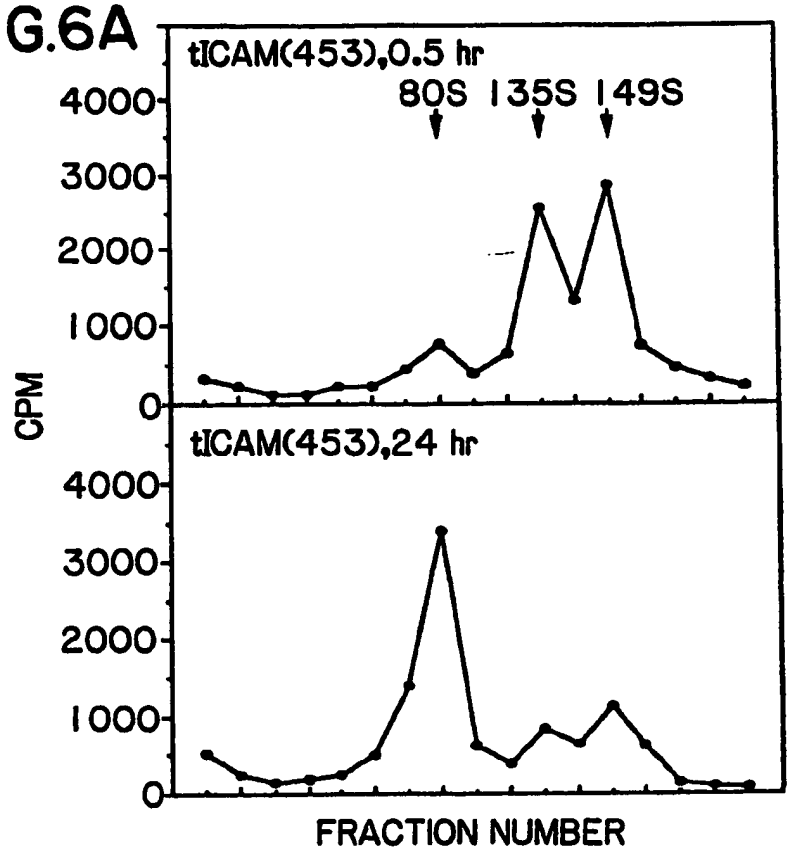

tICAM(453),0.5 hr
80S 135S 149S
4000
3000
2000
1000
0
CPM
tICAM(453),24 hr
4000
3000
2000
1000
0
FRACTION NUMBER

FIG.6B

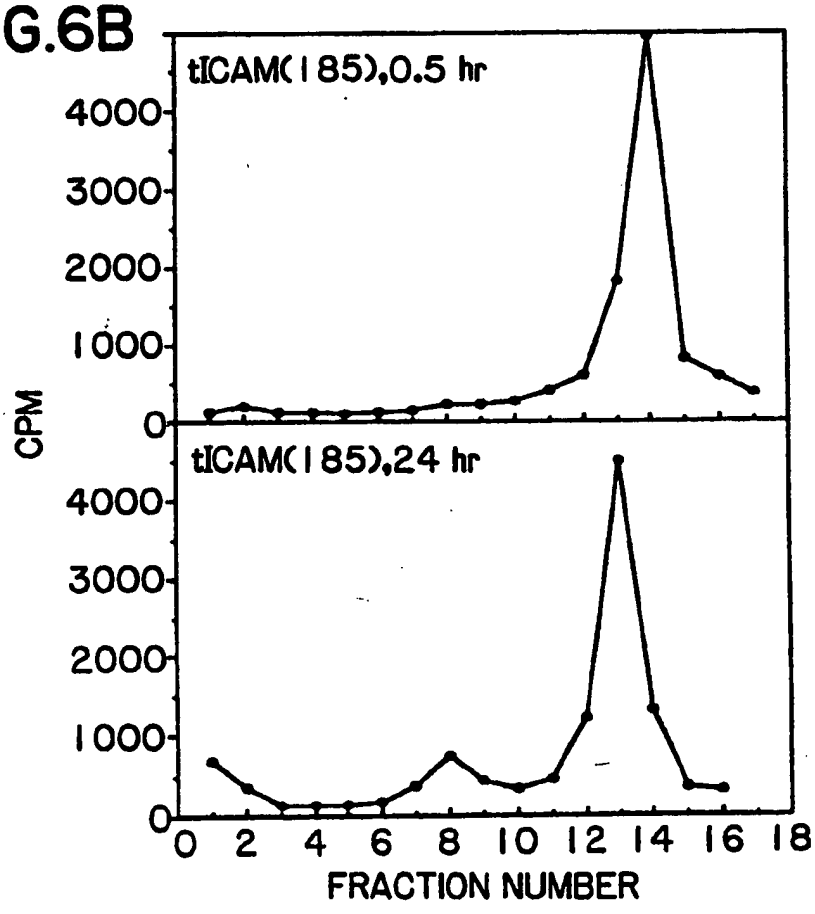

tICAM(185),0.5 hr
4000
3000
2000
1000
0
CPM
tICAM(185),24 hr
4000
3000
2000
1000
0
0 2 4 6 8 10 12 14 16 18
FRACTION NUMBER

FIG.6C

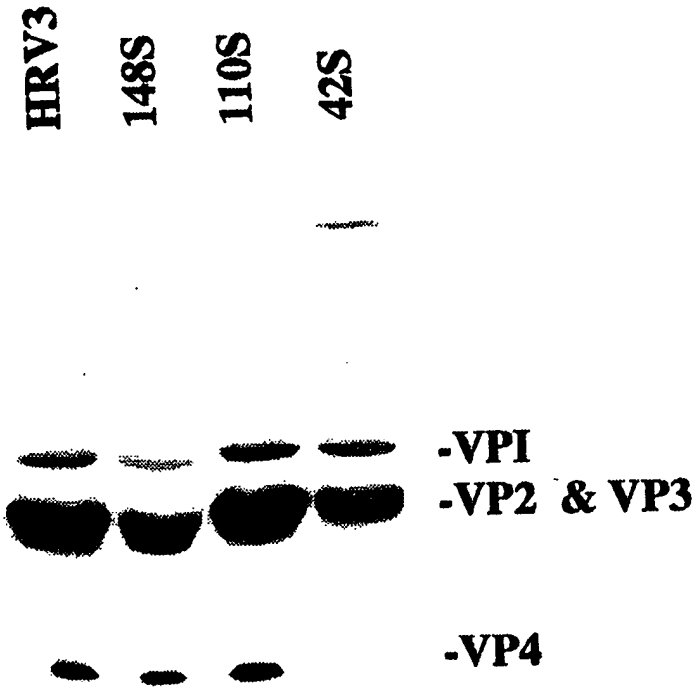


FIG.6D

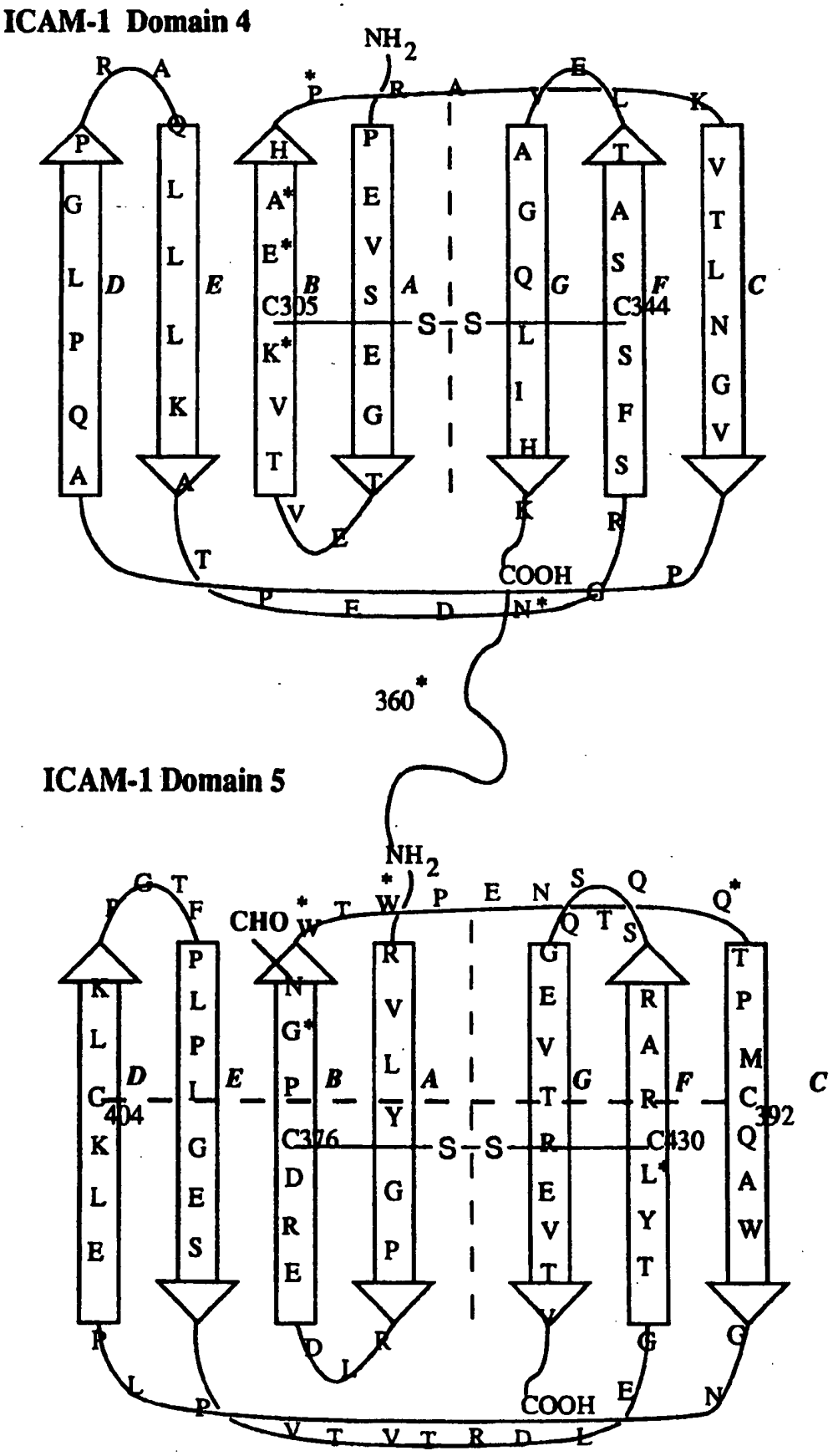


FIG.7

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International applicati n N .
PCT/US93/05972

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 7/00, 9/00, 17/00; A61K 37/02, 47/30
US CL :530/395, 402, 403; 514/2, 8, 12; 424/88
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/395, 402, 403; 514/2, 8, 12; 424/88

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

—

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Automated Patent System ("APS") database.
DIALOG database, files 155, 399, 5, 351.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Immunology, vol. 137, issued 15 August 1986, R. Rothlein et al., "A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1", pages 1270-1274, see entire article.	1-43
Y	Cell, vol. 61, issued 20 April 1990, D.S. Staunton et al., "The arrangement of the immunoglobulin-like domains in ICAM-1 and the binding sites for LFA-1 and rhinovirus", pages 243-254, see entire article.	1-43

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date f mailing f the international search report
04 AUGUST 1993	06 OCT 1993
Name and mailing address f the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized fficer THOMAS CUNNINGHAM
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05972

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Virology, vol. 58, issued 1986, J.E. Tomassini et al.. "The isolation of a receptor protein involved in attachment of human rhinoviruses", pages 290-295, see entire article.	1-43